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Genetic evaluation of haploid clonal lines of a single donor plant of *Populus maximowiczii*

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Summary. Haploid clonal lines derived from anthers of a single donor plant of *Populus maximowiczii* were evaluated for several quantitative traits. In a nursery test, variances due to clonal lines ranged from 8% to 12% of the phenotypic variance in growth cessation and flushing date, respectively. No variance in relative shoot growth rate was associated with clonal lines. In a greenhouse study, gametoclonal variance in several leaf morphology traits ranged from 9% to 37% of the total variance. In relative wood density, variation due to haploid lines accounted for 25% of the total variance. In an isozyme analysis of 20 haploid and dihaploid plants, significant non-Mendelian segregation in isocitrate dehydrogenase was detected. The implications of these results for tree breeding are discussed.

Key words: Anther culture – Dihaploids – Isozyme analysis – Gametoclonal variation – Tree breeding

Introduction

The number of reports describing haploid induction in woody angiosperms through anther culture is steadily increasing. In these studies, the manipulation of the medium, hormonal concentrations and treatment of the donor plant or explants are emphasized to increase anther response. While the ability to produce haploids at high frequencies in many genotypes is of importance, the necessity to genetically evaluate the induced haploids is of equal significance. One of the most important questions is whether induced haploids represent the gametic array of the donor or whether selection during culture eliminates a portion of the gametes, reducing the genetic base of the haploids. For example, non-Mendelian segregation was reported in alleles encoding phosphoglucomutase in anther-derived broccoli (*Brassica olearacea* var 'italica') (Orton and Bowers 1985). In contrast, in rice (*Oryza sativa*), haploids originating from genotypes with double heterozygous unlinked genes (i.e., waxy vs. unwaxy and liguleless vs. liguled) fit the 1:1:1:1 genotypic classes, suggesting segregation and independent assortment of these markers (Chen et al. 1983).

Plant breeders must also know the level of genetic variation in haploid or dihaploid plants if these are to be used in breeding programs. However, evaluations of the variation in quantitative traits in induced haploid genotypes are lacking in forest tree species and are not common in many agricultural crops.

Variation in quantitative characters (traits with a continuous distribution) among haploid plants of the same donor plant (i.e., among gametoclones) has been observed, but rarely statistically evaluated. Initial differences in leaf shape and vigor in anther-derived plants of several poplar species have been reported, but rarely quantified (Ho and Raj 1985; Chen 1987; Uddin et al. 1988). In older, better developed trees, heights ranged from 20 to 80 cm in 1-year-old *P. simonii* gametoclones (Wu et al. 1981) and from 19.5 to 131 cm in rubber tree (*Hevea brasiliensis*) gametoclones (Chen 1987). Surprisingly, in the latter species, some anther-derived plants had significantly greater diameter growth when compared to the donor tree (Chen 1987). In barley (*Hordeum vulgare*) gametoclonal variation accounted for 29% and 66% of the total variation in single plant yield and final height, respectively (Powell et al. 1986). Thus, there is evidence for substantial variation due to segregation,

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crossing-over and possibly the culture process itself, as discussed by Evans et al. (1984).

The usefulness of gametic-derived haploids for crop improvement is based on the assumption that homozygous dihaploid plants can be derived from them through chromosome doubling (Vasil 1980). These can occur spontaneously (Jensen 1974) or be induced by C-mitotic agents such as colchicine. However, mutations are often associated with such treatments (Jensen 1974), leading to inflated levels of genetic variation in the dihaploids. Therefore, estimating the magnitude of the genetic variance is more reliable when haploids are used prior to chromosome doubling.

The objectives of this study were to evaluate (a) the variation in some quantitative traits in haploid clonal lines of a single donor plant (*Populus maximowiczii* clone MD-1) and (b) the segregation pattern in haploid and dihaploid plants based on a heterozygous locus of the donor plant.

Materials and methods

Plant material

Fourteen haploid plants ($n=19$) and ten plants with the somatic chromosome number were selected from plants induced as described elsewhere (Stoehr 1989; Stoehr and Zsuffa, in press). These 24 test plants each originated from a different anther of *P. maximowiczii* clone MD-1. At the time of selection (April 1987), the plants were growing in a greenhouse and were about 1 year old. Clonal lines of the anther-derived plants were established by rooting greenwood cuttings in a sterilized 2 : 1 : 1 (v/v) mixture of peatmoss, perlite and vermiculite. All plants growing in the greenhouse were fertilized weekly with 20-20-20 N-P-K (Soluble Fertilizer Concentrate, Plant Products, Bramelea, Ont.) at a rate of 3 g/l (60 ppm N).

Test establishment

In August of 1987, three ramets each of the 14 haploid clonal lines were outplanted in a nursery bed in Toronto, Canada at a spacing of 30 cm × 30 cm in a completely randomized design (CRD) with single tree plots to evaluate bud break, height growth and growth cessation. Plants with the somatic chromosome number were not included in the evaluation of quantitative traits.

In one study, flushing date, growth cessation and height growth were evaluated. For this study, in the spring of 1988, the surviving 6 haploid lines with three ramets and 2 lines with two ramets were evaluated, a reduction from 14 lines and three ramets/clone due to winter mortality. Flushing date (FD), defined as the first day a leaf tip was visible in the terminal bud, was recorded in early April. At this time the height of each ramet was also measured and again at monthly intervals. Starting at the beginning of August, heights were measured at weekly intervals to determine growth cessation (GC). GC was defined according to Weber et al. (1985) as the first week in which final height did not change for three consecutive weeks. Height growth was evaluated as relative shoot growth rate (RSGR) to account for the initial size differences of the entries of the test population (Evans 1972). RSGR was calculated as follows:

$$RSGR = (\ln ht_{t2} - \ln ht_{t1})/t2 - t1$$

where ln = natural log, ht_{t2} = height of ramet at time of growth cessation, ht_{t1} = height at time of flushing, $t2 - t1 = 1$ as measurements were based on one growing season.

In another study of leaf morphology and relative wood density three ramets each from eight haploid lines and two ramets each from five haploid lines were assessed in the greenhouse. Ramets were grown in plastic tubes at a spacing of approximately 15 cm × 15 cm in single tree plots arranged in a CRD. No supplemental light was given during the initial 6 months and, therefore, ramets stopped growing in November 1987. In January 1988, the ramets were cut back to the third bud (as counted from the base of the plant) and put under artificial lights to extend the daylength to 16 h. After 5 months leaf and relative density measurements were taken from the shoots sprouting from the top axillary bud.

Leaf width (LW) at the widest point and the number of teeth on one side of the leaf (NT) were recorded on five fully expanded leaves on each ramet. Further, the length of the petiole (PL) and the internodal length (IL) were measured.

Relative wood density (WD) was determined by dividing the sprouting shoot into four equal-length sections. The unlignified top section of each shoot was discarded. In each of the remaining three sections the gravitational center was determined. From these centers, a 2-cm-long section was cut. These pieces were stripped of their bark and then split down the middle to remove the pith. Mean relative density was then determined using the average of the two samples per section calculated according to the "Maximum Moisture Content Method", after Smith (1954), a technique especially suitable for small pieces of wood.

Statistical evaluation

Ramet means for all variables with the exception of WD were subjected to one-way anova for the calculation of variance components. Coefficients associated with mean squares due to gametoclones were calculated according to Becker (1984) for unequal number of ramets and were found to be 2.7 and 2.8 for nursery and greenhouse variables, respectively.

RD was analyzed with a nested anova, based on the following linear model:

$$Y_{ijk} = u + L_i + G_j + L \times G_{ij} + R/G_{(j)k} + e_{(ijk)}$$

where Y_{ijk} = the mean relative density of samples in location i of gametoclone j and its ramet k; u = the overall mean; L_i = the effect of location i on RD; G_j = the effect of gametoclone j on RD; $R/G_{(j)k}$ = the effect of ramet k in gametoclone i; $e_{(ijk)}$ = the random error and/or the L × R/G interaction.

All statistical analyses were carried out on a microcomputer using the SAS package (SAS Institute Inc. 1982).

Isozyme analysis

A known heterozygous locus coding for IDH-2 (isocitrate dehydrogenase) in the donor plant (Stoehr 1989; Zsuffa 1988) was assayed in fresh root tips as described by Rajora and Zsuffa (1989) collected from rooted cuttings of the donor plant and in the 14 haploid gametoclones. Additionally, 10 plants with the somatic chromosome number derived during anther culture were included. Mendelian segregation of the alleles in the gamete-derived plants was evaluated using the Chi-square statistic.

Results

Phenological and growth variables

Mean flushing dates of haploid lines ranged from April 17 to April 22, 1988 (Table 1). This source of variation ac-

Table 1. Means (standard errors) of observed traits in haploid anther-derived clonal lines of *Populus maximowiczii* clone MD-1

Gameto-clone no.	No. ramets	Flushing date[a]	Growth cessation	RSGR[b] (cm/cm per year)	Leaf width (mm)	Petiole length (mm)	Internode length (mm)	No. of teeth	Relative density[c] ($g\,cm^{-3}$)
9	3	N/A	N/A	N/A	61.1 (2.2)	25.6 (1.3)	25.3 (1.2)	47.7 (1.6)	0.32
11	3	17.7 (0.7)	3.7 (0.3)	1.5 (0.2)	63.9 (2.3)	26.7 (1.5)	22.2 (1.7)	63.7 (1.8)	0.36
13	3	17.0 (0.0)	3.3 (0.9)	1.4 (0.1)	59.2 (1.4)	24.6 (1.6)	24.9 (2.1)	47.5 (2.1)	0.28
30	2	17.0 (0.0)	2.5 (0.5)	1.4 (0.2)	57.3 (2.2)	23.3 (1.3)	28.3 (1.2)	48.4 (1.2)	0.34
79	2	22.0 (3.0)	3.5 (0.5)	1.7 (0.5)	58.4 (2.7)	23.7 (1.7)	23.8 (1.9)	51.3 (2.2)	0.34
82	2	N/A	N/A	N/A	58.1 (3.0)	24.7 (2.6)	24.8 (2.2)	46.6 (4.2)	N/A
85	3	N/A	N/A	N/A	64.1 (0.8)	29.1 (1.6)	27.3 (1.3)	60.1 (2.4)	0.33
90	3	19.0 (0.0)	2.7 (0.9)	1.3 (0.3)	64.9 (2.4)	28.1 (2.4)	25.1 (1.2)	59.3 (3.7)	0.33
91	3	17.7 (0.7)	3.7 (0.9)	1.3 (0.2)	56.3 (2.3)	23.7 (1.7)	20.9 (1.4)	52.2 (1.7)	0.33
105	3	18.7 (1.7)	1.7 (0.7)	1.5 (0.3)	57.9 (1.3)	20.8 (1.4)	22.3 (1.5)	44.4 (2.0)	0.35
113	2	N/A	N/A	N/A	61.7 (2.7)	26.4 (2.1)	27.1 (1.3)	55.6 (3.4)	N/A
121[d]	3	20.3 (2.4)	2.7 (0.3)	1.8 (0.3)	49.5 (2.4)	18.9 (1.9)	23.7 (1.2)	52.9 (1.6)	0.36
122	3	N/A	N/A	N/A	58.9 (1.8)	22.1 (1.7)	27.6 (1.4)	49.1 (2.3)	0.35
Means:		18.6	2.9	1.5	59.6	24.5	24.8	52.3	0.34

N/A, Not applicable
[a] Flushing date counted as days from April 1, whereas growth cessation was recorded as weeks from August 28
[b] Relative shoot growth rate
[c] Standard errors for relative density were all <0.01 with the exception of clone 121, where is was 0.02
[d] Only two ramets used for leaf and relative density measurements

Table 2. Analyses of variance and estimated components of variance (VC) for observed traits in haploid gametoclones of *Populus maximowiczii* clone MD-1

Response variable Source of variation	*df*	MS	VC	VC (% of total variance)
Flushing date				
Gametoclones	7	7.24	0.7	11.7%
Error	14	5.33	5.3	88.3%
Growth cessation				
Gametoclones	7	0.271	0.020	8.4%
Error	14	0.217	0.217	91.6%
Relative shoot growth rate				
Gametoclones	7	0.04	N/A	0.0%
Error	14	0.11	0.11	100.0%
Lead width				
Gametoclones	12	40.45	3.1	8.9%
Error	23	31.90	31.9	91.1%
Petiole length				
Gametoclones	12	21.45	2.6	15.6%
Error	23	14.14	14.1	84.4%
Number of teeth				
Gametoclones	12	100.22	22.4	36.7%
Error	23	37.54	37.5	63.3%
Internode length				
Gametoclones	12	14.94	2.1	19.1%
Error	23	8.93	8.9	80.9%

counted for 12% of the total variance. The remaining variance was environmental, and probably inflated due to some uncontrollable shading effects in the nursery.

Ramets of the gametoclone that ceased growth the earliest stopped growing in early September, while ramets of the longest growing gametoclone stopped growing 2 weeks later (Table 1). Despite this range, the gametoclonal variation in GC accounted for only 8.4% of the phenotypic variance. Certainly the high levels of within gametoclonal variation accounted for this low proportion. The small number of ramets per gametoclone were possibly the cause of the high error variance.

No variation in RSGR was associated with gametoclones (Table 2), indicating that genes for this polygenic trait did not segregate during meiosis and/or assorted independently, thus nullifying their effects.

Leaf morphology variables

Mean LW ranged from 49.5 to 64.9 mm, accounting for 9.6% of the phenotypic variance (Tables 1 and 2). In comparison, 15.6% of the total variance in PL and 36.7% in NT were due to gametoclones (Table 2), as clone means ranged from 18.9 mm to 29.1 mm in PL and in NT from 44.4 to 63.7 (Table 1). IL ranged from 20.9 mm to 28.3 mm (Table 1), accounting for 19.1% of the total variance (Table 2). This type of variation, affecting crown architecture, and gametoclonal variation in petiole length may lead to differences in solar radiation interception,

Table 3. Analysis of variance and estimated variance components (VC) for relative density measurements of 11 haploid gametoclones growing in a greenhouse and derived from anthers of *Populus maximowiczii* clone MD-1

Source of variation	*df*	Mean square	VC[a]	VC (% of total variance)
Location (L)	2	0.0301	N/A	N/A
Gametoclone (G)	10	0.0041	0.00029	24.8%
L × G	20	0.0004	0.0	0.0%
Ramets/G	21	0.0016	0.00038	32.5%
Residual	42	0.0005	0.0005	42.7%
Total	95			100.0%

[a] Variance components were calculated as follows: VC for G: $(MS_G - MS_{R/G})/3k$; VC for L × G: $(MS_{L\times G} - MS_e)/k$; VC for R/G: $(MS_{R/G} - MS_e)$ 3 where k = 2.9, calculated according to Becker (1984) due to unequal number of ramets per gametoclone

already observed in poplar species (Isebrand and Michael 1986).

Relative density

Relative good density ranged from 0.28 to 0.36 g cm^{-3} among gametoclones (Table 1). These values are comparable to the mean specific gravity of coppice sprouts of the donor plant of 0.30 (Anderson et al. 1985).

The variance due to gametoclones accounted for 25% of the phenotypic variance (Table 3), indicating effective selection for this important trait.

Isozyme analysis

The isozyme assay of IDH-2, a heterozygous locus in the donor plant, revealed that six of the ten plants with 38 chromosomes were dihaploid, as determined by a single (homozygous) band. The four heterozygous plants presumably originated from the anther wall or connective tissue. The deviation from the expected 1 : 1 segregation of all examined plants with either a fast or slowly migrating allele was statistically significant at the 99% level (calculated Chi-square value = 7.2). The observed significant deviation from the 1 : 1 Mendelian segregation ratio in the gene encoding for IDH-2 points to a restricted gametic array in our haploid plants. A deviation from an expected Mendelian ratio in a marker gene has previously been observed by Orton and Bowers (1985) in anther-derived plantlets of broccoli (*Brassica oleracea* var 'italica').

Discussion

Quantitative traits

The variation among haploid lines is mainly caused by genetic differences inherent in the gametes of clone MD-1, but may also be confounded with culturally induced variation. Unlike in conventional clonal tests where the variation among clones is considered of purely genetic origin, the variance among gametoclones in this study may also include culture-induced variation (Evans et al. 1984). For example, mutation rates in tissue exposed to in vitro culture conditions exceed mutation rates in nature by 1000fold (Meins 1983), possibly leading to variation that is analogous to somaclonal variation in somatic tissue (Larkin and Scowcroft 1983). This culture-induced variation is known to increase with the length of exposure to and strength of growth hormones in the nutrient medium. Thus, the calculated variance among haploid lines may be somewhat inflated and, therefore, may represent the upper limit of the genetic variation.

Separating purely genetic sources of variation from culture-induced variation is not possible in gametoclones derived from a donor plant resulting from an outcrossing mode of reproduction. Gametes from such a genotype are very heterogeneous and most, if not all, of the variation among gamete-derived plants is a manifestation of the heterozygosity of the donor plant. Only an analysis with plants derived from a purely homozygous donor would give an estimate of the amount of culture-induced variation. Until these estimates are available for a tree species, we consider the variation among gametoclones to be of purely genetic origin.

The low levels of gametoclonal variation detected in this study could be expected as all plants in our study were closely related, originating from a single donor genotype. Low levels of variation in our samples may also point to negligible variation caused by the culture process. If the variance-inducing culture effect would have been large, variation among haploids would have been greater.

The potential value of haploid plants in tree improvement programs is in dihaploid (homozygous) parents producing hybrid offspring. Divers dihaploid parents will produce very heterozygous progenies, and heterozygosity is one possible source of hybrid vigor. Furthermore, desirable traits of different donor plants could be combined via hybridization more efficiently if dihaploids are used in the crossings. Dihaploids breed true, resulting in a greater chance for successful combination and expression of desired traits.

There is evidence from this study and others that in anther-derived plants isozyme alleles do not follow the Mendelian rule of segregation. Although the sample size for the analysis was small (14 haploid and 6 dihaploid lines) and only one heterozygous locus could be detected in the donor, the results give an indication of the selection which takes place at the microspore/pollen level for haploid/dihaploid plant production. IDH-2, possibly linked to a deleterious gene, may be subjected to selection during in vitro culture at the cellular level. According to

Bonga et al. (1988), this could cause the removal of recessive deleterious genes from anther-derived plantlets, thus improving the quality of the resulting genotypes. On the other hand, the resulting gametic plants are not representative of the gametic array of the donor plant, which could lead to an undesired restriction in a future breeding gene pool. The isozyme analysis also showed its usefulness in separating spontaneously arising dihaploids from somatic diploids. Without isozyme analysis, dihaploids are likely rejected unless other genetic markers indicate their gametic origin.

In conclusion, in the traits studied, gametoclonal variation in haploid plants derived from a single donor appears limited. Our isozyme data suggest that selection during the formation of plants from pollen/microspore reduces the array of alleles in the anther-derived plants.

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