POTENTIAL FOR INDIRECT SELECTION OF 'RESCUED' JACK PINE EMBRYOS BASED UPON LINKAGE BETWEEN SEEDLING DWARFISM AND MEGAGAMETOPHYTE ALLOZYMES

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Abstract.--Seedlings grown from seed collected from jack pine "witches brooms" segregate in a 1:1 ratio for dwarfism. Techniques are described for growing seedlings from this seed after the megagametophytes have been removed for allozyme analyses. Preliminary results indicated that there is linkage between the 6PG-2 isozyme locus and the dwarf trait, demonstrating the feasibility of indirect selection of "rescued" embryos based on megagametophyte allozymes. This is the first report of a linkage assignment for a gene controlling a morphological trait in forest trees.

Additional keywords: Pinus banksiana, isozyme analysis, witches' brooms, dwarfism, segregation, genetic linkage, recombination fraction, Br gene.

In the past, genetic studies of forest trees primarily involved quantitative traits. Trees are difficult to breed, have extremely long generation times, and, until recently, appeared to have few simply inherited qualitative traits. Inbreeding studies have revealed in conifer seedlings a number of morphological variants that appeared to be controlled by simply inherited recessive genes (e.g., albinos, dwarfs, and fused cotyledons) (Rudolph 1966, 1979; Franklin 1970). In addition, a dominant gene may be responsible for the dwarfing condition observed in the progenies of some witches' brooms in conifers (Fordham 1967; Johnson et al. 1965a,1965b, 1968a, 1968b; Johnson 1969; Waxman 1975). In jack pine (Pinus banksiana Lamb.) brooms of genetic origin (i.e., bud sports) produce seed but no pollen, and their offspring segregate 1:1 for dwarf and normal phenotypes. However, detailed study of these and other simple morphological markers has been severely constrained by the limitations inherent in tree breeding and by the deleterious phenotypic expression of these genes. In other plants, such traits have provided the basis for extensive gene maps and have yielded a wealth of genetic information.

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In the last 10 years, gel electrophoresis has enabled researchers to reveal the existence of a great deal of simply inherited genetic variation in conifers enzymes (Adams and Joly 1980, Eckert et al. 1981, El-Kassaby et al. 1982, Guries and Ledig 1978, O'Malley et al. 1979T Allozymes (multiple molecular forms of a gene or genes that code for the same enzyme) segregate 1:1 in megagametophytes from the seeds of individual open-pollinated conifers and provide a source of genetic markers that are well-defined and have little apparent effect on progeny viability. More than 60 allozyme loci may now be assayed in conifer megagametophytes using electrophoretic techniques (Ledig and Conkle 1983, Wheeler and Guries 1982). In corn and tomatoes, allozymes have been shown to have potential usefulness as markers for indirect selection, both in terms of increasing yield (Stuber et al. 1982) and labelling closely linked genes that are difficult to assess directly (Tanksley and Rick 1980, Tanksley 1983). These methods depend on the availability of well-defined gene markers.

Morphological gene markers have not previously been included in conifer linkage studies although additional genes could be helpful in extending the conifer gene map(Conkle 1981). Large numbers of gene markers are needed if indirect selection based on allozymes is to become feasible in conifers. Allozymes are more easily assayed in megagametophyte tissue than in foliage tissue due to technical difficulties in working with foliage tissue (Mitton et al. 1979). Analyzing megagametophytes normally involves destroying the germinating embryo from each seed. At times it is desirable both to sample the megagametophyte storage tissue and to grow the embryo. However, the seeds of many conifers, especially those from witches' brooms, are small and the developing seedling would have to be "robbed" of its entire nutritive megagametophytic covering in order to obtain sufficient tissue for electrophoresis. In this paper, we describe techniques for the "rescue" of jack pine embryos whose partially depeleted megagametophytes have been used for electrophoresis. These techniques allowed us to obtain joint segregation data for megagametophyte allozymes and a dominant dwarf seedling mutant from witches' brooms. Both allozymes and the seedling mutant segregate 1:1 in open-pollinated families, thus controlled breeding was not required.

MATERIALS AND METHODS

The witches' broomed trees used in this study originated within 40 km of Rhinelander in Oneida County, Wisconsin, except for Tree No. 2685, which was located about 20 km east of Grayling in Crawford County, Michigan. All of the brooms were on lateral branches except on Tree No. 2170, which had a broom in a terminal position on the main stem. Cones produced on the brooms were generally serotinous so cones containing seeds could be collected at any time of the year.

Cones were collected in 1980. Twenty cones per tree (10 from the normal portion of tree crowns and 10 from witches' brooms) were sampled for cone and seed size, seed yield, and seed quality. Cone size (volume) was determined by immersion in water containing a wetting agent in a graduated cylinder. The cones were dipped in boiling water up to 30 seconds to melt the resinous bonds binding the tips of the cone scales. The cones were dried in a circulating air oven at 55°C until the cone scales were reflexed, usually about 48 hours. Seeds were extracted by removing the cone scales by hand. Proportion of filled seed per cone was determined

by X-radiography on Kodak Type M film at 15 kVp, lmA, and exposure for 90 seconds. Seed size (volume) was measured by pouring the clean seed into a graduated cylinder.

The seed was germinated on moist perlite in petri-dishes in an incubator at 29°C using a continuous photoperiod. Germination usually began on the fourth day. Numerous preliminary trials were made to determine the optimum age and stage of development of the new germinants that would result in their highest survival when planted after removal of the megagametophyte for allozyme analysis, but before too much of the megagametophyte was absorbed by the cotyledons to permit allozyme analysis.

The germinants were planted into Tinus bookplanters containing 350 cc of a 2 peat:l perlite:l vermiculite mix. Each container was top-dressed with a 0.5 cm thick layer of finely sifted peat to facilitate planting of the small germinants. Each germinant was provided a high humidity environment by covering it with a 19 mm diameter glass vial. Identity of each germinant was maintained in the greenhouse at Rhinelander, Wisconsin, where they were grown through the spring and summer, 1981. Segregation for dwarfism in the seedling progenies was scored upon completion of this growth period. Corresponding identity of each megagametophyte was maintained during allozyme analyses in the Department of Forestry, University of Wisconsin-Madison, to permit determination of joint segregation ratios for dwarfism and polymorphic allozyme loci.

The electrophoretic methods used in this study are described in O'Malley et al. (1980) except for the stain for phosphoglycerate kinase (PGK), described by Siciliano and Shaw (1976). The statistical methods for analyzing segregation data are described in Nordheim et al. (1983).

RESULTS

Cones and seeds from normal and broomed portions of the tree crowns of four of the original broomed trees were available for analysis. Cones and seeds from grafted ramets of four other brooms were analyzed (table 1). Normal cones were not available from the grafted brooms. On the average, cones from brooms were only 38 percent as large as cones from normal portions of the tree crowns. Seeds from brooms were less than three-fourths the size of normal seeds on the average, but relative size of the two types varied widely between brooms. Total seeds per cone from brooms were half those from normal cones, but filled seed yields per cone from brooms were less than half those from normal cones. Percent of seeds filled in cones from brooms ranged from 5 percent in Tree No. 2170 to 82 percent in Tree No. 2685. Over all trees, the percent of filled seed in cones from brooms was about the same as in cones from normal crowns, but percent of filled seed was higher from grafted ramets of brooms than from original brooms on the ortets. Germination of filled seed, regardless of origin, averaged more than 90 percent. The low yields of seed in cones from brooms mean that at least twice as many broomed as normal cones must be collected to obtain comparable sample sizes for analysis. The smaller size of the seeds from broomed cones indicates that special care must be taken to preserve as much as possible of the megagametophyte for allozyme analysis.

Table 1.--Cone and seed size, seed yield, and seed quality from normal and witches'broomed jack pine.

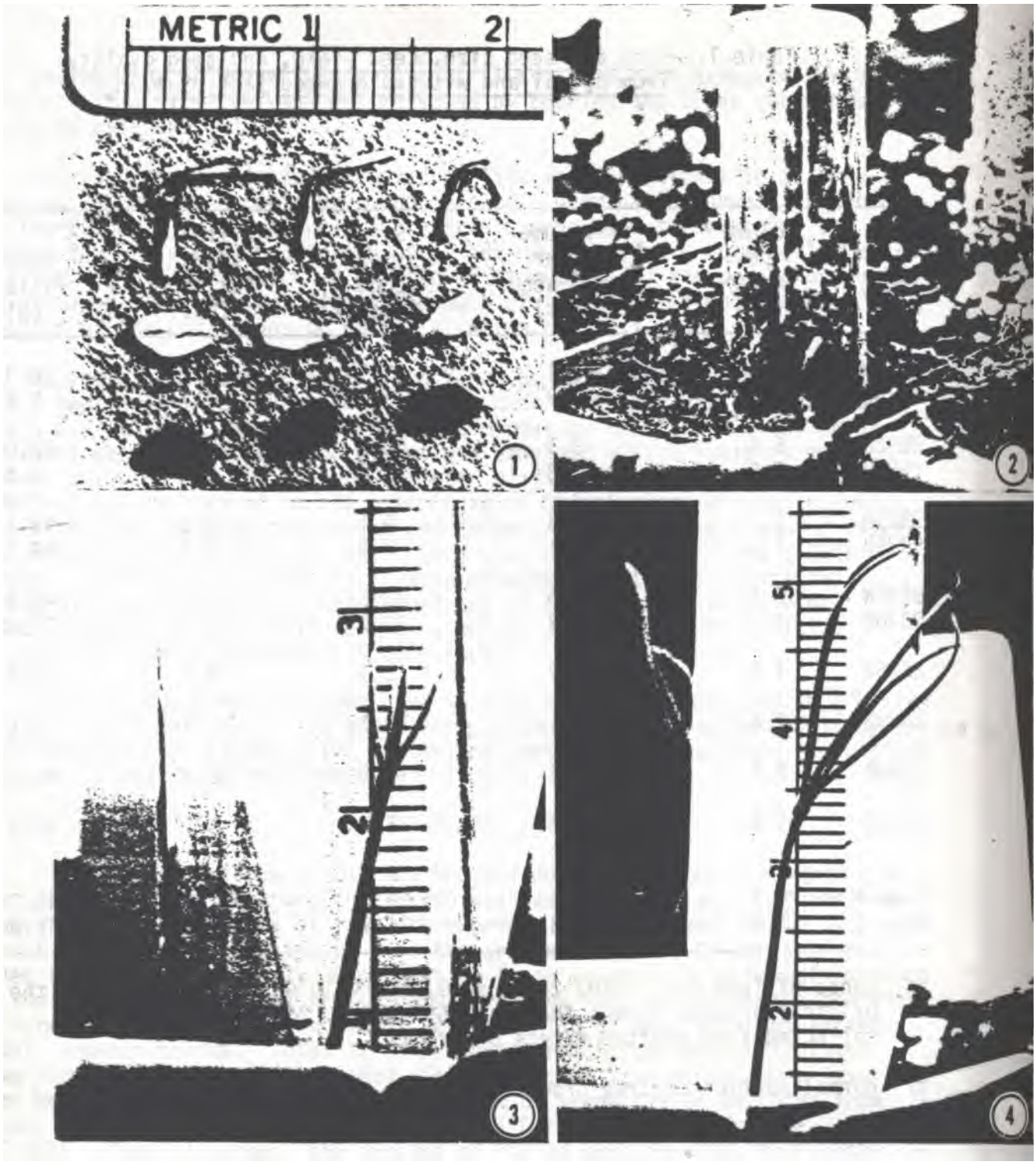
Tree No.[a]	Volume per cone (cc)	Volume per 1,000 seeds (cc)	Seeds per cone (No.)	Filled seeds per cone (No.)	Seeds filled (%)
2170N[b]	3.4	6.1	23.7	7.2	30.3
2170B[c]	2.4	2.9	13.2	0.7	5.3
2692N	6.6	8.1	45.8	23.8	52.0
2692B	2.0	3.3	23.6	2.0	8.5
9929N	2.2	6.3	9.1	4.2	46.2
9929B	1.5	5.1	7.5	5.2	69.3
9930N	6.5	11.5	33.5	15.0	44.8
9930B	1.9	7.7	20.7	7.0	33.8
2685B	1.2	9.2	12.0	9.9	82.5
2689B	1.6	5.1	10.7	5.9	55.1
2690B	2.2	6.2	9.2	6.1	66.3
2691B	1.8	6.8	15.7	5.7	36.3
Mean N	4.7	8.0	28.0	12.6	43.3
Mean B	1.8	5.8	14.1	5.3	44.6

a/ Cones of Tree Nos. 2170, 2692, 9929, and 9930 were collected from the original broomed trees; those of 2685, 2689, 2690, and 2691 were collected from grafted ramets of brooms.

b/ Normal portion of tree crown.

c/ Witches' broom.

Megagametophytes removed from new germinants 7 to 8 days after seed sowing still retained enough tissue for allozyme analysis (fig. 1). At this time the average total length of the germinants was 1.3 cm. The best survival and least damage was obtained when we used our fingers and fingernails to dissect the germinants. Extreme care was necessary to avoid



Figures 1-4.--1. Germinanted embryos (top), their removed megagametophytes (center), and seed coats (bottom). The germinants were planted at this stage and the megagametophytes were analyzed for allozymes. 2. Germinant without megagametophyte under glass vial immediately after planting in a bookplanter. 3. One-week-old seedling under glass vial. Cotyledons have elongated about 5 mm. 4. Two-week-old seedling. Cotyledons have reached maximum length and primary leaves are emerging. Protective glass vial has been removed.

dessication of the germinants during the dissecting procedure. Performing the operation on several layers of absorbent paper saturated with water kept the germinants from drying. They were immediately planted into the moistened media and covered with a protective glass vial (fig. 2). The planters were watered as needed to keep the media moist. Within one week, cotyledon elongation was clearly evident (fig. 3); at about two weeks cotyledons were fully elongated, and primary leaves were emerging. The glass vials were removed at that time (fig. 4). When we used these procedures, survival of the germinants averaged more than 86 percent.

The dwarf seedlings could be reliably distinguished from normal seedlings, after 6 months of growth. Six of the eight families of seedlings from the brooms did not differ significantly from a 1:1 segregation ratio for normal and dwarf seedlings (table 2), but the segregations for all families combined departed significantly from a 1:1 ratio.

Table 2.--Segregation analysis for dwarf trait in seedlings from open-pollinated witches' broom seed from eight jack pine trees.

Tree No.	Normal seedlings (No.)	Dwarf seedlings (No.)	Chi-square (1 d.f.) for 1:1 segregation
2170B	24	32	1.14
2685B	28	38	1.52
2689B	32	29	0.15
2690B	25	43	4.76*
2691B	15	15	0.00
2692B	7	17	4.16*
9929B	28	38	1.52
9930B	21	30	1.59
Total	180	242	14.84
Chi-square total :		14.84	8.d.f.
Departure from 1:1:		9.11***	1 d.f.
Homogeneity :		5.73	7 d.f.

* Significant at 5% level.
*** Significant at 0.5% level.

Megagametophytes from normal and broomed branches of several trees were compared for allozymes encoded by 45 loci (table 3). No allozyme differences associated with the broomed condition were found. Seven loci were polymorphic and five of the brooms were polymorphic at least at one locus. Analysis of the joint segregation of the seven allozymes in megagametophytes with dwarfing in corresponding seedlings from five brooms (table 4) revealed a probable linkage between 6PG-2 and the dominant gene controlling dwarfing in three trees. A chi-square test rejected independent assortment of 6PG-2 and dwarfing in two of the three families (alpha= 0.05 and alpha= 0.005); the sample

Table 3.--List of 45 allozyme loci surveyed for differences in megagametophytes of seeds from normal and witches' broomed jack pine.

Monomorphic loci		
ACP-1	βGLU[a/]	PEP-1
ACP-2	G6P	PEP-2
ADH-1	GDH	PEP-3
ADH-2	GPT-1	PEP-4
ADH-3	GPT-2	PEP-5
ALD-1	GR	PGM-1
ALD-2	IDH	PGI-1
AAT-1	MDH-1	PGI-2
DIA-1	MDH-2	TPI-1[b/]
EST (4MUA)	MDH-3	TPI-2
αGAL-1	MDH-4	UGPP-1[c/]
αGAL-2	ME	UGPP-2
αGAL-3	MPI	
Polymorphic loci		
ACO	6PG-1	PGK-1[d/]
AAT-2	6PG-2	G2D
FUM		

[a/] β-Glucosidase
[b/] Triose phosphate isomerase
[c/] Uridine diphosphoglucose pyrophosphorylase
[d/] Phosphoglycerate kinase

All other abbreviations are from Wheeler and Guries (1982).

Table 4.--Joint segregation of seven megagametophyte allozymes with the dwarf seedling phenotype, where a, b, c, and d denote the number of gametes in each of the four linkage classes and r is the Bayesian estimate of the recombination fraction over trees using a noninformative prior.

Tree No.	Segregation data a	b	c	d	Total	Chi-square analysis, 1 d.f. allozyme	dwarf	linkage
					ACO			
2690B	12	19	11	24	66	0.24	6.06*	0.55
2691B	8	12	7	3	30	3.33	0.00	2.13
					P (r > 0.357) = 0.95			
					DIA-2			
2690B	6	15	11	12	44	0.09	2.27	1.45
					P (r > 0.297) = 0.95			
					FUM			
2690B	7	11	15	23	56	7.14**	2.57	0.29
					P (r > 0.357) = 0.95			
					G2D			
2685B	15	22	12	15	64	1.56	1.56	0.25
2691B	10	6	5	8	29	0.31	0.03	1.69
					P (r > 0.368) = 0.95			
					6PG-1			
2689B	14	13	17	16	60	0.60	0.07	0.00
2691B	6	7	9	8	30	0.53	0.00	0.13
9929B	6	10	3	10	29	0.31	4.17*	0.31
					P (r > 0.407) = 0.95			
					6PG-2			
2685B	13	30	15	8	66	6.06*	1.52	8.73**
2690B	8	26	15	16	65	0.14	5.55*	4.45*
2691B	7	9	7	6	29	0.31	0.03	0.31
					P (0.321 < r > 0.411) = 0.95			
					PGK-1			
2691B	10	7	5	7	29	0.86	0.03	0.86
					P (r > 0.278) = 0.95			

* significant at 5% level.
** significant at 1% level.
*** significant at 0.5% level.

size of the third family was smaller. Segregation for the dwarf character was distorted in family 2690B and for the allozyme in family 2685B, but the estimation of recombination fractions is not affected unless both loci are distorted. Bayesian and likelihood methods were used to estimate recombination fraction (r) and to evaluate significance because the linkage is loose and the parental/recombinant gene arrangements are unknwon (Nordheim et al. 1983).

The maximum likelihood and Bayesian (noninformative prior) estimates of recombination fraction for 6PG-2 and the dwarfing gene are 0.371 and 0.368, respectively. The confidence level of the largest Bayesian confidence interval excluding r = 1/2 is 99.9% and strongly supports linkage. This approximately corresponds to a conventional test with (alpha) = 0.001. Smith's Bayesian probability of linkage is based on the posterior probability distribution for recombination fraction incorporating 0.05 as the prior probability of linkage. It is moderately suppprtive of linkage, Prob. (r < 1/2) = 0.410. Thus, if the probability of this gene pair being linked was 0.05 before data were obtained, it is eight times larger after evaluating the segregation data from these three families. Finally, the z-score (common log of the ratio of the likelihood evaluated under r = r, and r = 1/2) was 1.833, indicating that linkage is almost 70 times more probable than free recombination. There is no reason to suspect heterogeneity of recombination fraction among the three families (likelihood ratio homogeneity chi-square is 0.52 with 2 df). These methods of evaluating linkage are approximate, but they do show that linkage between the dwarfing gene and 6PG-2 is probable. Additional data are desirable to confirm the linkage at the p(= 0.005 to 0.001 level for measures of significance other than the Bayesian confidence level (i.e., z-score 3 and Prob. (r< 1/2) >0.90).

DISCUSSION

The segregation of dwarf and normal seedlings from 6 of the 8 jack pine witches' brooms showed good agreement with the 1:1 ratio expected from a heterozygote for dominant gene. However, the segregation for all families combined departed significantly from a 1:1 ratio with more dwarf seedlings than expected. The slow growing dwarfs might be more likely to survive the 'rescue' techniques than normal seedlings.

Substantial variation is found both within and among the dwarf progenies of witches' brooms (Fordham 1967; Johnson et al. 1968a,b; Waxman 1975) suggesting a chromosomal or heterogeneous origin for the polymorphism. However, broom chromosome numbers are normal (discussion following Waxman 1969), chromosomal variants seldom segregate close to Mendelian ratios following meiosis unless the defect is small (Khush 1973), and known conifer aneuploids are not reproductive (Ching and Doerksen 1972; Johnson and Saylor 1972; Owens 1967). Copes (1975) found differential isozyme expression among "yewlike" and "twisted-needle" dwarfs and normal Douglas-fir. The "yewlike" dwarfs were similar in appearance to the aneuploids described by Owens (1967) and Ching and Doerksen (1972). In this study we found no abnormal broom allozyme patterns at 45 loci. The homogeneity of the linkage between 6PG-2 and the dwarfing character in three brooms suggests that the same gene may be affected in many or all cases. We tentatively designate this gene Br, a dominant gene causing a dwarf seedling phenotype and brooming in mature jack pine.

This study is the first (to our knowledge) to report a linkage assignment for a gene controlling a morphological trait in forest trees, It also demonstrates the feasibility of indirect selection of "rescued" embryos based on megagametophyte allozymes. For example, among the embryos whose megagametophytes carry the 6PG-2 slow allele, 64% will be dwarf compared to an expected 50% of unselected embryos. The efficiency of indirect selection increases as the recombination fraction between two genes decreases. In tomatoes, an allozyme marker tightly linked to a gene conferring nematode resistance (Tanksley and Rick 1980) facilites the transfer of resistance to other genetic materials through backcross breeding. Selection against other allozymes specific to the parent contributing the desired gene increases the efficiency of backcross breeding so that fewer generations will be needed to eliminate most of that parent's genome.

Tanksley and Rick (1980) point out that selection based upon allozymes makes backcross breeding options plausible for long-lived species. Allozyme markers for genes controlling resistance would provide significant advantages in resistance breeding of trees.

Single gene control of disease resistance mechanisms has been documented in conifers (e.g., western white pine, McDonald and Hoff 1971 and sugar pine, Kinloch and Littlefield 1977). For instances where disease resistance is controlled by a single gene, the possibility of linkage between such genes and allozyme markers may make screening for resistance a much faster process. The genetic diversity of trees carrying rare resistance genes could be increased through these methods. Not only would generation times be reduced over conventional backcross methods, but the requirement for screening would be postponed to the last generation and the numbers of individuals to be carried per generation would be substantially smaller so that it may be practical to use accelerated growth techniques (e.g., Wheeler et al. 1982) to turn over generations more quickly. More efficient methods would allow a larger number of resistance mechanisms to be manipulated. However, single gene resistance can be lost (Kinloch and Comstock 1981) and breeding would be only one component of an integrated control program (McDonald 1979).

Allozyme frequencies change in response to selection for quantitative traits in maize (Stuber et al. 1980). Reciprocally, quantitative traits respond to selection for allozymes in the same maize population (Stuber et al. 1982). Indirect selection on allozyme gene frequencies appears to be more efficient in achieving gains than selection on the quantitative trait itself. However, it is uncertain whether the allozymes themselves respond directly to selection, or whether the changes in gene frequency are caused by linkages (i.e., "hitchiking", Hedrick 1982). Comparison of allozyme frequencies in selected and unselected tree populations may help to resolve this question. Indirect selection based on allozymes might provide a means to bring new genetic material into established tree improvement programs if such frequency differences can be documented.

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