cessfully screen for superior provenances. This conclusion is based on the fact that provenance by environmental interactions (when they exist) are because of scale differences rather than actual changes of rank. This would imply that results of test plots in one environment can be applied at other untested environments. Also, correlations among provenances at different ages are large enough to encourage the use of early results as predictions of future performance. For example, the correlation between height means for ages 5 and 20 years is 0.67. These correlations change little if adjusted rather than unadjusted means are used. The largest changes were from 0.46 to 0.32 between ages 2 and 20 years and from 0.75 to 0.67 between ages 9 and 20 years. All other correlations change by 0.03 or less. (The data at age 2 years were not adjusted because no attempt was made to measure nursery survival.)

Because of the uncertainties involved in evaluating provenances or genotypes, viewing early test results as a means of eliminating poor genetic material may be more profitable than selecting superior stock. The material that is not eliminated would form a mixture of a broadly based source of genetic material that could be used for reforestation. Although some theoretical loss in growth potential may occur by not using the best genotype, the difficulties in determining the best stock combine with the potential for ecological disaster from the use of genetic monocultures to argue against using a narrow genetic base for reforestation.

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New Methods to Prepare Squashes to Study Microsporogenesis in Pinus resinosa Ait. I. Formulas Based on Glycerin, Water and Dimethylsulfoxide

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Abstract

A new method was developed for the preparation of squashes of microstrobili of *Pinus resinosa* for the study of microsporogenesis. The tissues were fixed in ethanol-acetic acid (3:1 v/v), and stored in ethanol-glycerin-water (1:1:1 vlv). To 10 columes of glycerin-water-dimethylsulfoxide (2:1:7 v/v) (GWD), 1 volume methyl salicylate and 1 volume chlaral hydrate stock (20 g of chloral hydrate in 100 ml GWD) were added (GWDMC). The GWDMC was preheated to 170° C, the tissues were dropped into the hot GWDMC and kept at 170° C for 20—40 minutes. If the tissues were stained in carmine, the carmine was added to GWDMC before heating to 170° C. Toluidine blue and nigrosin did not tolerate heating to 170° C, and tissues were stained in these at room temperature after the GWDMC treatment was completed. Cell separation was excellent. Meiotic prophases were well cleared and showed details of chromosomes well. In the stages from metaphase to tetrads, the cytoplasm was often somewhat contracted. Microspores were well cleared and stained.

Key *words:* Pinus resinosa, microsporogenesis, cytology, squashes, meiosis.

Zusammenfassung

Es wurde eine neue Methode zur Herstellung von Quetschpräparaten von Mikrostrobili auf der Basis von Glycerin, Wasser und Dimethylsulfoxyd bei *Pinus resinosa* AIT. entwickelt, durch welche insbesondere die Phasen der Meiose gut aufgehellt werden.

Introduction

The most commonly used fixative-stain for the preparation of squashes for karyological studies of conifer tissues is aceto-carmine. Because this fixative-stain has some drawbacks, an alternative method was developed using lactic acid, propionic acid, and dimethylsulfoxide as the carmine solvent (BONGA and VENKETESWARAN, 1976). Later, a new improved method based on glycerin, water, and dimethylsulfoxide was developed for root tip squashes of conifers, which allowed squash preparation at higher temperatures, (BONGA, 1977). However, subsequent investigations showed that although before diakinesis the chromosomes fixed, stained, and spread well, this method was not fully satisfactory for the study of microsporogenesis of conifers,

because from metaphase to the end of the tetrad stage, the cytoplasm generally failed to clear; it contracted, and clumped the chromosomes.

In most plant species, the meiocytes are surrounded by a relatively impermeable callose wall from the late prophase till the end of the tetrad stage (Heslop-Harrison, 1966). This suggests that the observed contraction and non-clearing of the cytoplasm was caused by callose inhibiting the penetration of the glycerin-water-dimethyl sulfoxide-carmine into the cells. All further experiments therefore, were designed to try to: 1) reduce the callose by dissolving it, or to disrupt it by physical means, and 2) increase the capacity of the stain formula to clear the cytoplasm but without damaging the chromosomes.

Material and Methods

In the spring of 1976 and 1977, male reproductive shoots of *Pinus resinosa* Ait. were collected three times a week from about one week before the onset of meiosis until pollen shedding. While the microstrobili were still small, they were transferred whole to the fixative; after they had started to expand they were cut in small pieces before fixing. After 24-hours fixation at room temperature, most specimens were transferred to a storage fixative composed of ethanol-glycerin-water, (1 : 1 : 1 v/v) (EGW) (Gerlach, 1969), and stored at room temperature until further processing; others received various treatments (see later) before being stored in EGW. Most material was stained directly after EGW storage but some of the material received further treatments (see later) before being stained. The specimens were stained in 1 ml of preheated stain in 4-ml screwcap vials (Bonga, 1977) and were kept at the required constant temperature in an oven placed inside a fume hood.

After staining, small tissue samples were squashed in a drop of stain under a coverglass using light needle pressure. All microscopic examinations and photography were carried out in green light (Kodak Wratten B filter 58). If upon examination, squashes showed insufficient flattening or staining, they were left (sometimes with a weight on top of the coverglass) at room temperature from several hours to days to achieve additional flattening of the cells and to intensify staining of the chromosomes.

Fixatives

The following fixatives were tested: 1) Ethanol-acetic acid, (3 : 1 v/v) (EA); 2) Ethanol-acetic acid-chloroform (6 : 1 : 3 and 6 : 3 : 1 v/v), (These chloroform containing fixatives (Carnoy) were chosen because it was considered possible that chloroform, a powerful organic solvent, could change the permeability of the callose.); 3) Ethanol-propionic acid (3 : 1 v/v); 4) Isopropanol-acetic acid (3 : 1 v/v); 5) 2,2-Dimethoxypropane-acetic acid (3 : 1 v/v), Dimethoxypropane reacts rapidly with water to form methanol and acetone (Lin, Falk, and Stocking, 1977) which could make it useful as a component for fixatives.); 6) Ethanol-acetic acid-ethylene glycol monoethyl ether (3 : 1 : 1 v/v), (Ethylene glycol monoethyl ether is often used as a dehydrating agent, is a solvent for esterwax (Grimstone and Skaer, 1972), and is used in industry as a solvent for a wide range of products. On this basis, it was assumed that it might change the permeability of the callose.); and 7) An ammonium oxalate containing macerating fixative recently developed by Sharma, *et al.* (1977).

Treatments to Dissolve Callose during Storage

Material fixed in EA was transferred to the storage fixative EGW in combination with various chemicals. The following chemicals were used, each being dissolved in 100 ml of EGW: 1) 1 g potassium hydroxide; 2) 3 g calcium chloride; 3) 3 g zinc chloride; 4) 1 g ammonium oxalate. Potassium hydroxide, calcium chloride, and zinc chloride solutions are solvents for callose (Currier, 1957), and ammonium oxalate has a macerating effect (Sharma, *et al.* 1977). After 30—60 days of treatment at room temperature, the specimens were transferred to fresh EGW.

Treatments after Storage, but before Staining

Some samples were taken from EGW and hydrolysed at 70° C for 1 or 4 hours in dilution series of hydrochloric acid, formic acid, or trichloracetic acid, using water or EGW as the diluent (0.25, 0.5, 1, 2, and 4 ml acid in 10 ml diluent). Tissues were also heated in dilution series of potassium hydroxide (0.025, 0.05, 0.1, 0.2, and 0.4 g in 10 ml water or EGW). Other samples were heated in methanol-chloroform (1 : 1 v/v) at 45° C for 2 hours or overnigth, and then returned to EGW for a few hours. Such a treatment was used by Borstel and Lindsley (1959) to clear and partially remove cytoplasm from cells.

Water and sodium chloride solutions have been used for separating and spreading meiotic chromosomes (Beçak *et al.* 1977). Therefore, the effect of soaking the tissues in sodium chloride solutions (0.025,, 0.05, and 0.1 g in 10 ml water) and glycerin-water combinations (2 : 1, 1 : 1, 1 : 2, 1 : 4, and 1 : 8 v/v) was determined.

It was attempted to break the callose and achieve selective destruction of the cytoplasm by physical means. The tissues were boiled in water (boiling water has been used to fix and uncoil chromosomes (Sharma and Sharma, 1972)), or water and glycerin 4 : 1 v/v, or EGW. It was also attempted to rupture the callose by freezing and thawing the tissues or by repeated short exposures to vacuum.

Staining and Squashing

Initially the staining and squashing methods were similar to those developed earlier (Bonga, 1977) with glycerin-water-dimethylsulfoxide (2 : 1 : 7 v/v) (GWD) being the solvent for the stain Carmine (c*) (2.5 g of c in 200 ml GWD), and staining being carried out at 160° C for 40—60 min. To determine if carmine could be replaced by other stains, 32 different nuclear stains were tested. For this purpose, 0.1 g of each stain was dissolved in 20 ml of GWD. However, most of the stains were heat sensitive and could not be used at 160° C. For all heat-sensitive stains the procedure was modified in that the tissue was heated in the solvent without stain, i.e. GWD or modifications thereof, and subsequently stained at room temperature in GWD with stain.

To determine if cell separation, cytoplasm clearing, and chromosome separation could be improved, and primarily if cytoplasm collapse in the later phases of meiosis could be reduced, preliminary experiments were carried out in which several chemicals commonly used in cytology were added in various concentrations to GWD. The only chemicals that showed promise were methyl salicylate (M) (used by Boke (1970) for clearing and storage of tissues), chloral hydrate (C) (it dissolves membranes (Ballou, *et al.* 1974) and has been used to clear cytoplasm, to remove exine, and to stain pollen (Rupert and Webster, 1972)), and ammonium oxalate (O) (used by Sharma, *et al.* (1977) to macerate tissues). Therefore, the effect of these chemicals was determined more precisely. M was tested at 0.5, 1, 2, and 4 ml added to 10 ml GWD. Twenty grams of C were dissolved in 100 ml GWD and 0.25, 0.5, 1, and 2 ml of this stock solution was added to 10 ml of GWD before testing. Three grams of O were dissolved in 100 ml GWD, and 0.3, 1, 3, and 9 ml of this stock solution were added to 10 ml of GWD.

To determine the optimal temperature for cell separation, cytoplasm clearing, and chromosome preservation, tests were caried out using constant temperatures at 10° intervals between 110 and 180° C for 20, 40, 60, and 120 minutes. To heat the tissues as rapidly as possible, a screwcap vial containing about 1 ml of solvent, with or without stain, was placed for about 15 minutes in an oven set at the required temperature. Then the tissue was taken from the EGW storage fixative, placed in the hot solvent, and heated for the required length of time.

* All stains are abbreviated with small letters, all other chemicals by capital letters.

In a separate experiment, exposure to constant temperatures at 10° intervals between 200 and 270° C for 10, 20 and 30 minutes was tested. Since the solvents boiled at temperatures over 200° C the following procedure was used at these temperatures. A vial with solvent was preheated to 170° C, the tissue was added to the hot solvent, the vial was sealed inside a pressure bottle and exposed to the 200° C or higher temperatures for the required length of time.

Results

Of the fixatives tested, those containing chloroform were the least satisfactory. The one containing ethylene glycol monoethyl ether slightly reduced the degree of cytoplasmic collapse but also coarsened the image of the chromosome. Very little difference was observed between any of the other fixatives, and therefore, EA, one of the fixatives most often used in plant cytology, was used in all further work.

None of the chemicals added to the storage fixative EGW was effective in reducing the collapse of the cytoplasm and in improving clearing of the cytoplasm. Therefore in all subsequent work, the tissues were, after fixation in EA, stored in EGW without chemical additions.

Of the treatments after storage, but before staining, only a few had some beneficial effect. Heating the tissues in methanol-chloroform (1 : 1 v/v) for 2 hours sometimes cleared the cytoplasm of the meiocytes and somewhat reduced its collapse without apparent damage to the chromosomes. However, the treatment was not consistently effective, and therefore, was not included in later routine procedures.

Treatment in glycerin-water was ineffective, except if the combinations 1 : 4, or 1 : 8 v/v were used. Keeping the tissues in these for several hours at room temperature before staining often resulted in breaking the cell walls of prophase meiocytes, thus freeing nuclei and chromosomes from cell wall constraints and aiding chromosome separation and flattening.

Of the efforts to disrupt the callose and/or the cytoplasm by physical means, only prolonged boiling in EGW had a beneficial effect. If boiled till the tissues turned dark and the boiling liquid dark brown and syrupy, the cytoplasm disintegrated leaving darkly stained and well-separated, but somewhat swollen, chromosomes for observation.

Adding methyl salicylate to the basic stain solvent GWD improved cell separation and flattening, and adding chloral hydrate improved clearing of the cytoplasm. The optimal combination (GWDMC) was 10 volumes GWD, 1 volume methyl salicylate, and 1 volume of chloral hydrate stock solution. Ammonium oxalate, 1 ml stock solution, added to 10 ml GWD improved chromosome separation, but the improvement was not consistent in repeat experiments, and therefore, this chemical was not included in later routine procedures.

Best cell separation, flattening, clearing of cytoplasm, and chromosome separation were obtained if the tissue was heated in GWDMC at 170° C for 20 or 40 minutes. Longer exposure resulted in a slight swelling and sometimes fusion of the chromosomes. Short exposure to temperatures higher than 170° C resulted in cell and chromosome separation comparable to that at 170° C, but with reduced stainability. Longer exposure to such high temperatures was destructive.

Carmine in GWDMCc could be replaced by orcein or Giemsa and heated to 170° C, resulting in the chromosomes being stained less intensely than if stained in carmine. Staining also occurred with Evans blue, chlorazal black, Chicago blue, azure blue, nigrosin and toluidine blue 0, but none of these stained at temperatures as high as tolerated by carmine, i.e. with these the tissues had to be treated in the solvent without stain at 170° C and then stained in solvent with stain at room temperature. The most satisfactory stain within this group was toluidine blue 0 (Fisher Scientific Cy, C.I. No. 52040) dissolved in GWD, hence referred to as GWDt. Second best was nigrosin (Fisher Scientific Cy, C.I. No. 50420) dissolved in GWD, and referred to as GWDn. All the others overstained the cytoplasm and cell walls.

Generally, chromosomes stained darkly in the carmine stain GWDMCc. However, if microscopic examination showed the chromosomes to be understained, extra staining could be achieved by sucking a drop of GWDt or GWDn under the coverglass with filter paper, or by lifting the coverglass and adding a small drop of GWDt or GWDn to the tissue.

Microscopic Observation

In squashing, the diploid tisues of the microsporophylls broke into small clumps of cells or single cells. This liberated large numbers of intensely stained meiocytes which spread out well under the coverglass *(Fig. 1)*.

During the early phases of meiosis, parts of the thread-like chromosomes separated reasonably well from the main mass of chromosomes. Darkly stained beads (chromomeres) were clearly discernible along the chromosomes *(Fig. 2)*. Pairing of chromosomes and chromomeres was sometimes observed in the zytogene *(Fig. 3)* and pachytene stages. During diplotene, the darkly stained contracted chromosomes had a prickly appearance, and chiasmata and repelling of the homologues were clearly visible *(Figs. 4* and *5)*. Generally, the nuclear membrane of the early meiotic stages was destroyed during preparation of the squash, but sometimes it remained intact *(Fig. 5)*.

Whereas the cytoplasm in meiocytes in their early stages of meiosis partly or completely disintegrated during the staining procedures, this cytoplasm often remained intact in later meiocytes. From diakinesis onward, contraction of this cytoplasm often presented a problem; it tended to clump the chromosomes, even under repeated needle tapping and pressure. In the few meiocytes in which the cytoplasm had partly or completely disintegrated during the staining procedures, the chromosomes were well separated and showed good structural detail *(Figs. 6 to 8)*. Microspores, on the other hand, were generally well cleared and stained and showed little cytoplasm contraction *(Fig. 9)*.

The cell walls sometimes broke during squashing, freeing the chromosomes from cell wall and cytoplasmic constraints, thus aiding in the separation of the chromosomes. In leptotene, zygotene and pachytene material this often resulted in partial unwinding of the ball of chromosomes *(Fig. 10)*; sometimes in more complete *(Fig. 11)*, and rarely in almost complete *(Fig. 12)* unraveling of the chromosome ball. As the ball unravelled a less obstructed view of the chromosomes was obtained, and details of structure showed better.

Breaking of the cell wall often also helped to separate and flatten diakinesis chromosomes. Chiasmata showed well and individual chromatids could be seen in the chiasmata as well as in other parts of the bivalents *(Fig. 13)*.

One or two large and several smaller nucleoli *(Figs. 1, 2, 11* and *12)* were frequently prominent up to diplotene. Two large nucleoli were often paired in the pachytene *(Fig. 14)*, and diplotene *(Fig. 15)*, i.e. fusion of the two nucleoli apparently was delayed or did not occur. Sometimes a non-

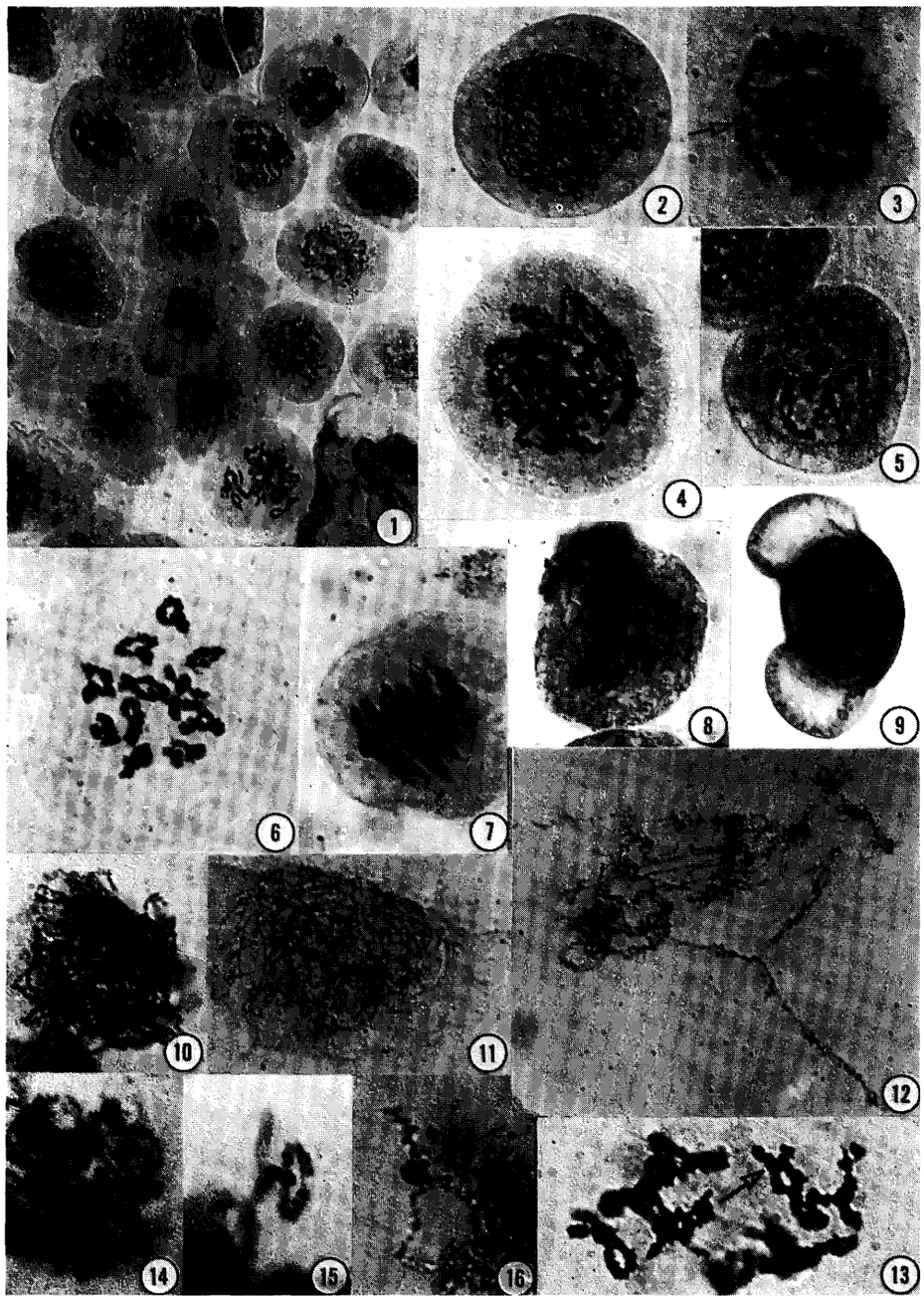

Figure Captions

Fig. 1. — Meiocytes in the early stages of meiosis. The microsporophyll tissues were broken into a large number of small clumps of cells and individual cells, freeing a large number of meiocytes. Stained in GWDMCc. × 250

Fig. 2. — Zygotene. Note dark chromomeres on chromosomes. Stained in GWDMCc. × 500

Fig. 3. — Zygotene. Note pairing of chromosomes and chromomeres (arrow). Stained in GWDt. × 750

Fig. 4. — Diplotene. Homologues have started to separate. Stained in GWDt. × 500

Fig. 5. — Diplotene. Nuclear membrane still intact. Stained in GWDn. × 500

Fig. 6. — Metaphase I with 12 bivalents. Stained in GWDMCc. × 625

Fig. 7. — Early anaphase I. Stained in GWDMCc. × 500

Fig. 8. — Late anaphase I. Stained in GWDt. × 500.

Fig. 9. — Microspore. Stained in GWDt. × 600

Fig. 10. — Free zygote nucleus. Partial unwinding of ball of chromosomes. Note darkly stained chromomeres. Stained in GWDMCc. × 1000

Fig. 11. — Free zygotene nucleus. More unwinding than in Fig. 10. Note at least 5 nucleoli. Stained in GWDt. × 875

Fig. 12. — Free zygotene nucleus. Almost complete unwinding of the ball of chromosomes. Note pairing of chromosomes, and one large and several small nucleoli. Stained in GWDt. × 750

Fig. 13. — Free diakinesis chromosomes. Note chromatids in chiasma area (arrow), and near chromosome ends. Stained in GWDMCc. × 1000

Fig. 14. — Paired nucleoli on pachytene chromosomes. Stained in GWDMCc. × 1000

Fig. 15. — Paired nucleoli on diplotene chromosomes. Stained in GWDMCc. × 1000

Fig. 16. — Nucleolus with non-stained nucleolar organizer (arrow). Note chromomeres on chromosome. Stained in GWDn × 1000

staining part of the chromosome (nucleolar organizer) could be seen to traverse the nucleolus *(Fig. 16)*.

Discussion

The techniques described above were well suited for study of the early stages of microsporogenesis in *Pinus*. For the later stages of meiosis, these methods were less effective because the cytoplasm generally remained intact, thus preventing spreading of the chromosomes. The various chemical treatments designed to dissolve the callose walls of the meiocytes did not improve removal of the cytoplasm. It is not known if these treatments failed to remove the callose walls, thus inhibiting contact between the hot stain solvents and the cytoplasm, or if the cytoplasm in the later meiotic stages was less sensitive to the solvents than the cytoplasm in earlier stages. This failure to disintegrate the cytoplasm resulted in clumping of the chromosomes.

The most effective treatment to disintegrate the cytoplasm, and separate the chromosomes in the later stages of meiosis was prolonged boiling in EGW; the main drawback of this method being swelling of the chromosomes. However, the results were sufficiently promising to start a series of experiments to determine if this boiling technique could be modified to one that would cause less chromosome swelling. These experiments will be discussed in the next paper.

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Seedling Growth Rates, Water Stress Responses and Root-Shoot Relationships Related to Eight-year Volumes among Families of Pinus taeda L.

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Summary

First-year seedling growth characteristics were correlated with mean individual-tree 8-year volumes of 16 North Carolina families of *Pinus taeda* L., with a view to developing seedling screening methods for early volume growth.

Rates of seedling height growth (mm/week) were influenced by family differences in seed size until the seedlings were about 140 mm tall. Thereafter, until bud-set, the growth rates of well-watered seedlings became positively correlated with 8-year volumes, on a mean family basis, especially for families field tested on a poorly drained site. For families tested on a better-drained site, the correlations with seedling height growth rates were significant only when the seedlings were subjected to mild water stress: families which produced the greatest 8-year volumes grew fastest under mild water stress as seedlings. These families also tended to have high root to shoot relative growth rates, as revealed by regressions of shoot on root dry weights of seedlings grown in two contrasting media. This suggested that superior volume-producers on the better-drained site avoided water stress by producing extensive root systems.

There was little evidence that superior volume-producing families had prolonged seasonal periods of growth as seedlings.

It seems promising to further examine seedling height growth rates as a measure of future family field performance, taking measurements after seed size effects have disappeared and under environmental stresses that mimic those which influence growth in the field.

Key words: bud-set, early genetic evaluation, juvenile-mature correlations, progeny-test, root-shoot relationships, water stress responses.

Zusammenfassung

Einjährige Sämlinge von *Pinus taeda* L. aus 16 Familien in North Carolina wurden im Wachstum mit 8 Jahre alten Kiefern aus den gleichen Familien verglichen. Hierbei wurde gefunden, daß bis zu einer Sämlingsgröße von etwa 14 cm eine Abhängigkeit zur mittleren Samengröße der jeweiligen Familie gegeben ist, während danach Standorteinflüsse, wie z. B. die Wasserführung des Bodens für den Holzzuwachs bestimmend sind. So waren später Beziehungen im Gesamtwachstum (Volumen) der Sämlinge zum mittleren

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