SOMACLONAL VARIATION IN HYBRID POPLARS FOR RESISTANCE TO SEPTORIA LEAF SPOT

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Abstract. --Tissue culture techniques have been used to obtain hybrid poplars with putative resistance to leaf spot caused by Septoria musiva from clones previously susceptible to the disease. Stem internode explants were used to obtain proliferating callus cultures. Adventitious bud formation and shoot proliferation were then induced. Elongated shoots were excised and rooted in a peat : perlite medium under high humidity. After acclimation, rooted plants were transferred to the greenhouse. Variant plants were selected among the regenerants using a leaf disc bioassay that rapidly distinguishes plants with high resistance. The incidence of somaclonal variation in disease resistance differed among the genotypes tested. Over 500 tissue culture-derived plants tested for resistance using the bioassay have been planted in the field and are being evaluated for field performance. Somaclonal variation and tissue culture have the potential to significantly change tree breeding programs by reducing the time required for selecting and improving desired traits.

Additional keywords: aseptic culture, Populus, disease, Septoria musiva.

INTRODUCTION

Recent advances in the manipulation of plant cells and techniques for aseptic culture have increased the prospects of developing improved trees in ways never before possible. Tissue and cell culture technologies provide the tools that make many new biotechnologies feasible. The current and potential applications of aseptic culture techniques to forest tree improvement have recently been reviewed by several authors (Sederoff and Ledig 1985, Durzan 1985, Miksche 1985, Haissig et al. 1987).

Aseptic culture techniques can be valuable tools for tree improvement programs by capturing additive and nonadditive traits. Perhaps even more important is the possibility that aseptic culture may be a new source of useful genetic diversity. Tissue culture has been used extensively in the past to clonally propagate plants, but useful genetic variation has been recently found to occur among regenerated plants. Passage of plant cells through a tissue culture cycle can result in increased spontaneous phenotypic and genetic variability. Somaclonal variation is the term used to describe variation exhibited by plants obtained from aseptic culture (Larkin and Scowcroft 1981).

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The potential application of somaclonal variation for crop improvement first became evident in work with sugarcane. Variation was observed in morphological, cytogenetic, and isozyme traits (Heinz and Mee 1971). Tissue culture was used to obtain variants of a sugarcane variety that were highly resistant to the Fiji virus disease (Krishnamurthi and Tlaskal 1974) and to the toxin produced by Helminthos orium sacchari, the cause of eyespot disease (Larkin and Scowcroft 1983).

Variants in potatoes regenerated from protoplasts were called protoclones, which had increased resistance to early blight caused by Alternaria solani and late blight caused by Phytophthora infestans in addition to several other characteristics (Shepard et al. 1981). The authors proposed incorporating several trait variations in a cultivar exhibiting the first desirable characteristic by using consecutive culture cycles.

Several authors have recently reviewed the history of somaclonal variation, its potential applications, and the use of tissue culture in crop improvement (Evans et al. 1984, Evans and Sharp 1986, Lorz 1984, Chaleff 1983, Wenzel 1985, Scowcroft et al. 1983, Orton, 1984).

Although the cause of somaclonal variation is not yet completely understood, the phenotypic and genetic variation found indicates that many factors are involved. Somaclonal variation can be preexisting genetic variation that is expressed in regenerated plants, or it can be induced by the tissue culture process itself (Scowcroft and Larkin 1983). Working with tomatoes, Evans and Sharp (1983) provided classical genetic proof that tissue culture can be mutagenic. The plant species, genotype, source of explant used for culture initiation, and perhaps most importantly, the duration of the culture cycle may influence the variability observed (Lorz 1984). Meins (1983) suggested that tissue culture induces cellular destabilization that can result in diverse heritable changes. In addition to epigenetic variation, tissue culture can cause genetic changes ranging from single base pair changes to chromosome deletions, translocations, and changes in chromosome number (Evans and Sharp 1986).

Somatic variation, now considered a general phenomenon, has been observed in a variety of agronomic crops. Evidence also was presented for variation in Populus. One growing season after planting, a wide variation in height, number of branches, and leaf traits was detected in trees from six Populus X euramericana clones regenerated from callus culture (Lester and Berbee 1977).

Somaclonal technology is a genetic engineering approach to plant improvement that is rapidly being applied to many agronomic crops to improve many plant traits, including disease resistance. Applying somaclonal technology to forest trees offers an even greater advantage because of the long generation times of trees and the opportunity to introduce desired traits not possible through traditional breeding.

In our study, we have used hybrid poplars to demonstrate the potential application of somaclonal technology for increasing the disease resistance in a tree species. Populus was chosen because of its worldwide importance as a

source of fiber and energy and its amenability to whole plant regeneration through a variety of cell and tissue culture systems. Additionally, potential biomass yields from hybrid poplar plantations are now seriously limited by the foliar and canker diseases caused by the fungal pathogen Septoria musiva Peck. Somaclonal variation has the potential to enhance the productivity of hybrid poplars by increasing disease resistance.

MATERIALS AND METHODS

Populus Clones

Stock plants of five hybrid poplar clones of varying resistance to S. musiva were propagated in the greenhouse from hardwood cuttings. The resistance of these clones to Septoria in field trials in Minnesota, Wisconsin, and Iowa has previously been reported (Ostry and McNabb 1985, 1986). The parentage and field resistance to S. musiva of these reference clones are presented in Table 1.

Tissue Culture

Unorganized callus cultures of the reference clones were initiated from stem internode explants taken from the greenhouse stock plants. Leaves were removed from 5-cm-long shoot tips, and the stems were rinsed for 15 sec. in 70% ETON. The stems were then placed in a beaker of 2.5% NaOCl containing 10 drops of Tween-20 per liter. After 10 min. the solution was decanted, and the stems received three 5 min. rinses in sterile distilled water. Stem internode explants 1 cm in length were excised from the shoot tips and placed horizontally into shell vials containing 10 ml of Woody Plant Medium (WPM) (Lloyd and McCown 1980) plus 20g l^{-1} sucrose, 6g l^{-1} Difco Bacto agar, and 2.3uM 2,4-D. Cultures were maintained in the dark in an incubator at 25C.

Three to four weeks after culture initiation, proliferating callus at the cut ends of the explants was excised and subcultured onto fresh WPM containing 0.45uM 2,4-D and returned to the incubator. Proliferating callus cultures were subsequently divided and subcultured onto fresh medium every 3 to 4 weeks. Callus cultures of clones ranged from 5 to 13 mos. old before shoot proliferation was induced.

Adventitious bud formation and shoot proliferation were induced by transferring callus cultures to WPM containing 1.1uM 6-benzylaminopurine (BA) and 0.27uM 1-naphthaleneacetic acid (NAA). Cultures were incubated under light (300-lux) with a 16 hr photoperiod at 25C.

Elongated shoots were excised and rooted in a peat:perlite medium under high humidity in a lighted growth room. After gradual acclimation to lower humidity, plants were transferred to the greenhouse (18-30C, 18 hr photoperiod).

Selection of Somaclonal Variants

Isolates of S. musiva were obtained from diseased hybrid poplar leaves by placing 5mm^2 pieces of leaves onto a potato dextrose agar medium and

Table 1. *Populus* reference clones and their susceptibility to *Septoria* in field trials.

Clone	Parentage	Disease resistance[a/]
NE 41	*P. maximowiczii* X *P. trichocarpa* 'Androscoggin'	M
NE 314	*P. nigra* var. *charkowiensis* X *P. nigra* var. *caudina*	H
NE 299	*P. nigra* var. *betulifolia* X *P. trichocarpa*	L
NE 319	*P. nigra* var. *charkowiensis* X *P. trichocarpa*	M
NE 293	*P. nigra* var. *betulifolia* X *P. nigra* Volga	H

[a/] H = no defoliation, M = premature defoliation in lower and mid crown, L = premature defoliation throughout crown

Table 2. Percentages of somaclones not reaching 50% necrosis.

Source Clone	Number of regenerated plants tested	Number and percent not reaching 50% necrosis
NE 41	23	12 (52)
NE 314	25	11 (44)
NE 299	157	48 (31)
NE 319	84	11 (14)
NE 293	90	27 (30)

incubating them at 20C in an incubator under continuous light (300 lux). To induce sporulation, cultures were later transferred to an agar medium containing 180ml/l V-8 juice and 2g/l CaCO3 and incubated under continuous light at 20C.

Conidia of S. musiva were removed from 7- to 10- day-old cultures by flooding them with distilled deionized water and gently agitating the plates. A conidial suspension was adjusted to a concentration of 1 x 10b conidia per ml of water. An in vitro bioassay (Ostry et al. in preparation), developed to identify poplars with resistance to S. musiva, was used to screen for variant plants. Leaves that had just fully expanded were collected from tissue culture-derived plants and from the parent stock plants (source plant) of each clone tested. All tissue culture-derived plants had been growing in the greenhouse for at least 1 month. Leaves were rinsed with distilled water, and 18mm diameter discs were extracted using a cork borer. Corresponding wells were removed from the 2% water agar in petri plates, and the leaf discs were placed into them abaxial surface up.

Twelve leaf discs from each plant were inoculated with 0.1 ml of the spore suspension, and two leaf discs served as uninoculated controls, receiving distilled deionized water only. Leaf discs were incubated in a continuously lighted (200 lux) growth room maintained at 20-25 C.

Disease progression was monitored by measuring the necrotic area on each disc every 2 days by using a dot grid (25dots/1.8cm^2). Measurements continued until the control leaf discs started to become necrotic.

Data Analysis

A regression analysis of the pooled measurements was performed with percentage necrosis and elapsed time as the dependent and independent variables, respectively. From this, the time elapsed to 50% necrosis and the rate at which 50% necrosis occurred were estimated. Based on these parameter estimates, the resistance to Septoria of the tissue culture-derived plants was compared to that of the parent plants.

RESULTS

Adventitious shoot proliferation from callus cultures varied by clone. Clone NE 299 yielded the greatest number of rootable shoots on the culture medium used. Previous work in our laboratory provided evidence that some Populus genotypes were more recalcitrant than others under identical culture conditions.

There were no obvious mutant phenotypes among the regenerated plants from any of the clones except for two plants with mutant leaves that did not root. All plants that rooted resembled their parent source plants.

Of the three Septoria-susceptible clones used in this study, (NE 41, NE 299, and NE 319), plants with increased resistance were recovered from two of them. Many regenerants from clones NE 41 and NE 299 were significantly more resistant to Septoria than their parent source plants.

The estimated necrosis-time curves for all the clones are presented in Fig. 1. The regenerants from clone NE 319 were as susceptible to Septoria as the parent plant. Regenerants from the two resistant clones (NE 293 and NE 314) were more than and as resistant as their parent plants, respectively.

A range in disease resistance was exhibited by the regenerants from all the clones (Fig. 2). The variation in resistance exhibited among the regenerants depended on genotype. Some of the regenerated plants were more susceptible or nearly as susceptible as the parent clone; however, some plants were clearly more resistant. Some of the leaf discs from these plants never did reach 50% necrosis, and several did not exhibit any disease symptoms (Fig. 3). The percentages of regenerated plants that were more highly resistant than their source plants are presented in Table 2.

There was no clear trend in the rate of somaclonal variation detected in regenerants from callus cultures of different ages. Variants with increased levels of resistance were recovered from callus of all ages.

DISCUSSION

Based on our results, it is apparent that tissue culture can be used in attempts to increase disease resistance in poplar clones. The putative resistant clones regenerated from tissue culture clearly reacted differently in the bioassay than their parent source plants. Because the cause for this variation is not known, these plants cannot be considered true mutants until this phenotypic change observed is shown to result from a permanent heritable genetic change. However, even a stable epigenetic variant of an elite poplar clone would be of value because poplars are usually vegetatively propagated.

We do not know if other traits besides their disease resistance have been altered in these plants. The desirable qualities of the clones must be retained, and the new trait must be expressed for somaclonal technology to be of any value. In addition, a change in the resistance to one pathogen may either increase or decrease the resistance of a clone to other pathogens or insect pests. This underscores the importance and need for field testing plant genotypes regenerated from tissue culture. The plants from our study have been planted and are being monitored for growth and disease resistance in the field.

Somaclonal technology is simpler to exploit than other types of genetic engineering. Scowcroft and Larkin (1983) have proposed that somaclonal variation generates mutant genotypes similar to those resulting from spontaneous or induced mutation, but at a much higher frequency. They believe that most of the genetic changes responsible for somatic variation are induced by the culture cycle itself. Somatic variation may be a better means for tree improvement than either direct gene insertion or Agrobacterium-mediated transformation because of the many technical difficulties that must be overcome with those methods.

In the work reported here, we screened regenerated plants. A far more powerful technique would be selection at the cellular level, favoring growth of only the variant cells resistant to disease. We are currently researching

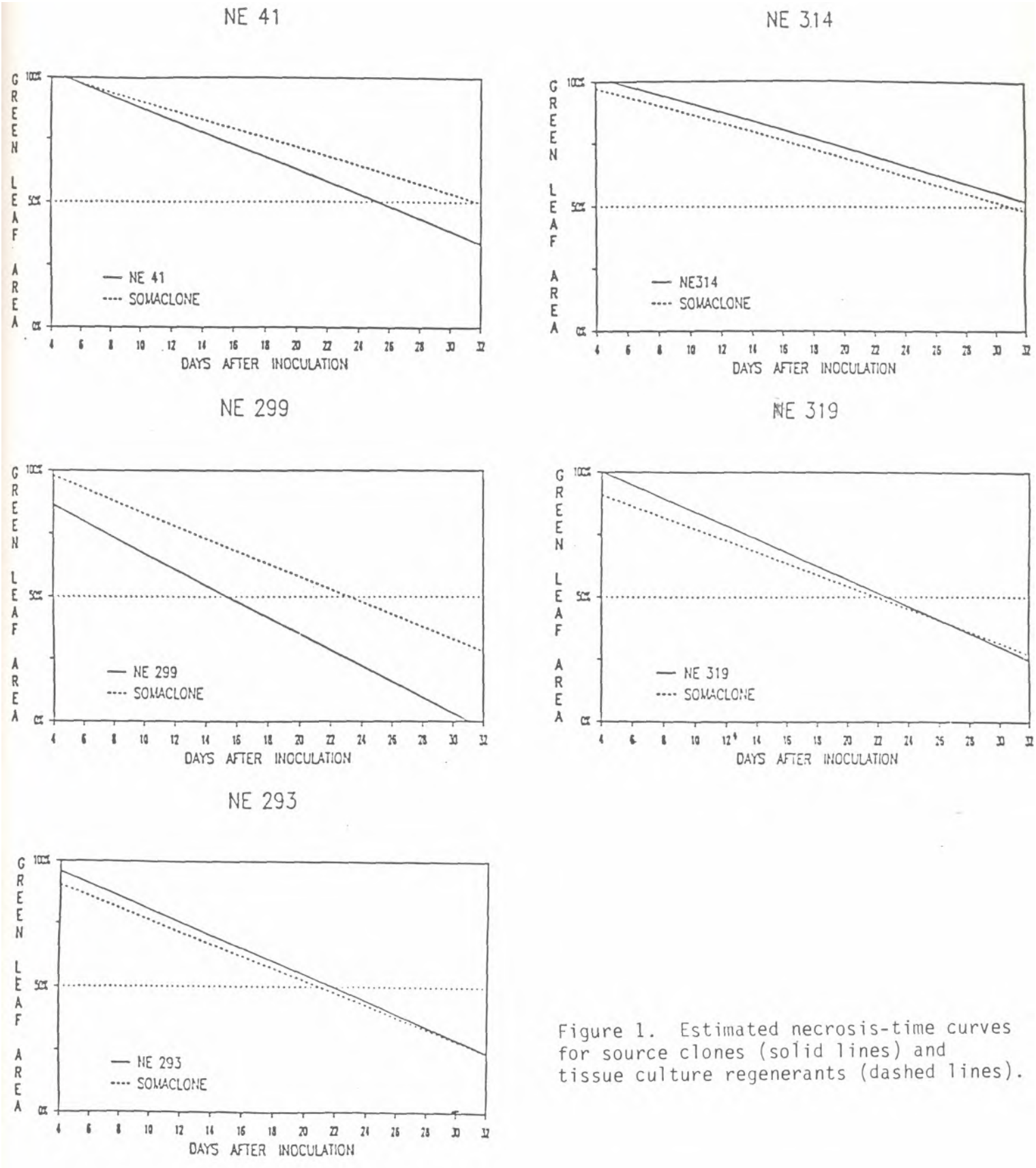

Figure 1. Estimated necrosis-time curves for source clones (solid lines) and tissue culture regenerants (dashed lines).

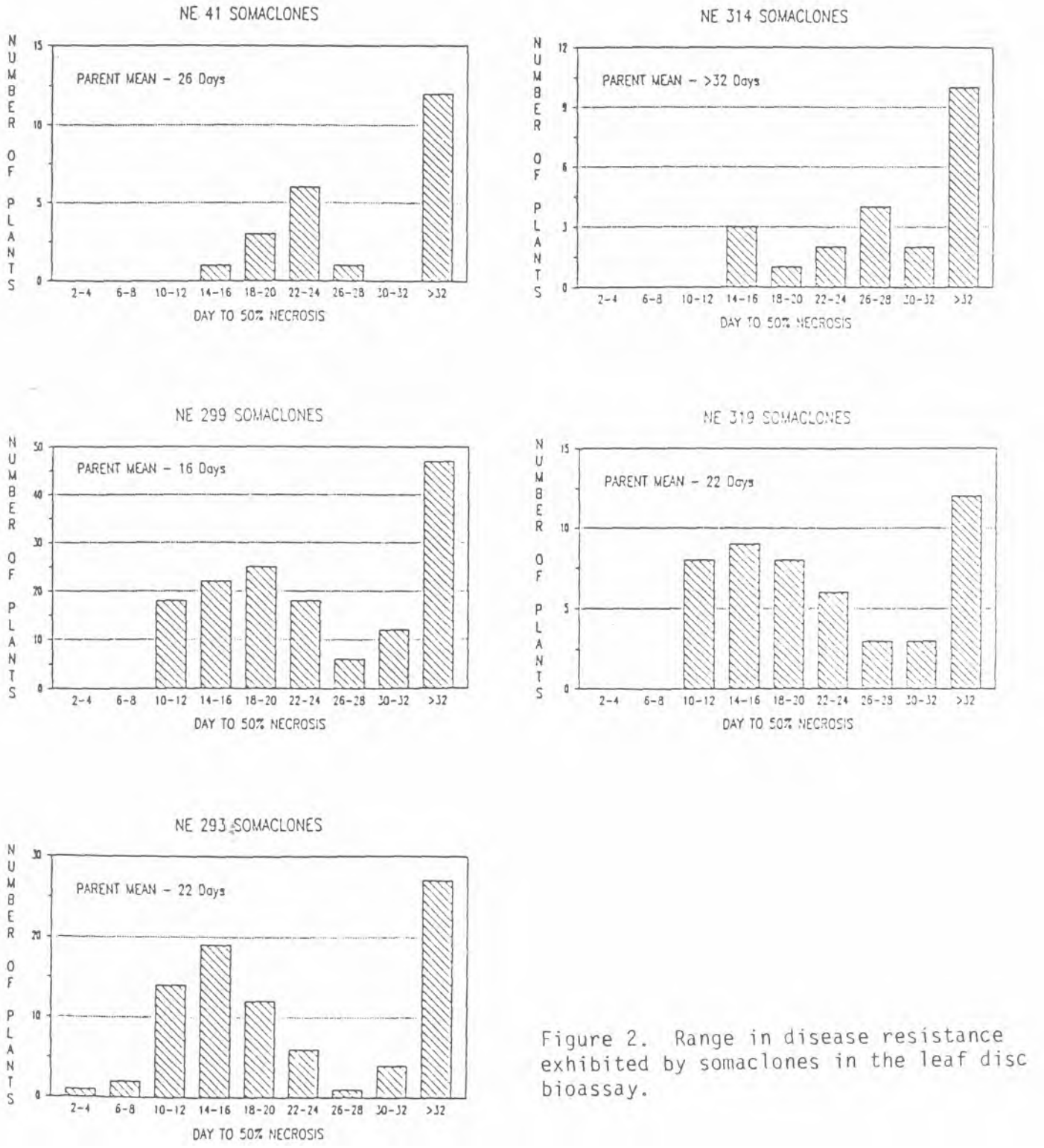

Figure 2. Range in disease resistance exhibited by somaclones in the leaf disc bioassay.

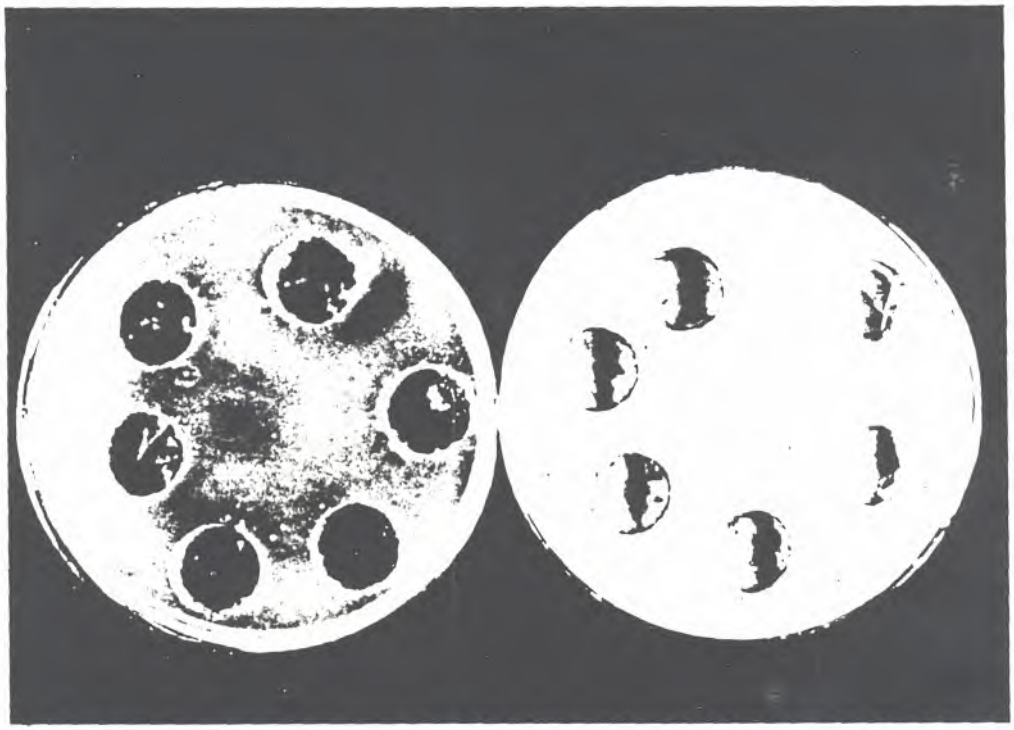

Figure 3. Disease severity on leaf discs from source plant (left) and regenerated somaclone (right).

techniques to use partially purified culture filtrates to screen for or select Populus cells that are resistant to S. musiva. The advantages and limitations of in vitro screening and selection for disease resistance have recently been reviewed (Daub 1986, Hammerschlag 1984). It is of primary importance that the desired trait be expressed or selectable in culture.

Somaclonal variation and cellular selection for disease resistance, coupled with early, rapid screening of regenerated plants is a new, more efficient technology for tree improvement. This technology needs to be incorporated into conventional breeding programs to meet both short- and long-term goals. Moreover, the ability to introduce new traits such as disease resistance into elite lines via somaclonal variation eliminates the segregation of other desirable characteristics that occurs in hybridization. In addition, we now have the potential in tree improvement to use somatic variation to respond more quickly to adaptive changes in pathogen populations.

The value of somaclonal variation in tree improvement remains to be proved. However, our results thus far indicate that Populus cell cultures may not be static and are amenable to manipulation. If the resistance to Septoria in the putative resistant clones we have identified is stable in the field, a substantial gain will have been made. Somaclonal technology may provide a valuable new option for the tree breeder and plant pathologist and should be incorporated into tree improvement programs and applied to other tree species.

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