USE OF TISSUE CULTURE TECHNIQUES IN A HARDWOOD TREE IMPROVEMENT PROGRAM

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ABSTRACT

Tissue culture per se is not a method of genetic improvement but is instead a method of vegetatively propagating trees and plants. Tissue culture methods available to forestry are micropropagation, organogenesis, and somatic embryogenesis. A long-term industry-state cooperative conventional tree improvement program has used the techniques of selection, hybridization and polyploidy to produce rapid-growing Populus species hybrids. Aspen hybrid seedling populations have been produced that at 18 to 20 years grow approximately twice as fast, have 20 to 30 percent longer fiber length, and, 8 percent higher wood density than widely used native aspen. Only modest improvement, has been made, however, in producing hybrids that are resistant to the regions most serious forest management problem, hypoxylon canker (Hypoxylon mammatum Wahl., Miller).

Recent Ph.D. and related research has resulted in tissue culture procedures that allow us to readily screen seedling populations for seedlings that are highly resistant at the cellular level to the canker toxin, the reported determining factor in the disease. Also utilized are procedures for bioassaying field tested parent trees, hybrids, and young seedling populations for resistance. Planned is an expanded tree improvement program that will combine conventional tree improvement techniques and tissue culture procedures to produce hypoxylon resistant hybrid clones and seedling populations. Micropropagation and organogenesis methods are presently available for use in producing operational clonal plantings. At present there appears to be adequate natural resistance in existing seedling populations, so that the use of more sophisticated genetic engineering techniques (transformation, protoplast fusion, etc.) may not be required to solve this serious disease problem. Tissue culture techniques similar to those described could be expected to be useful in evaluating experimental crosses and screening and generating disease resistant parent trees in other hardwood tree improvement programs.

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INTRODUCTION

Emphasis on the use of tissue and cell culture in the propagation of forest trees has increased dramatically in the last five to six years. The tissue culture methods available to forestry are micropropagation, organogenesis and somatic embryogenesis. Micropropagation is the in vitro propagation of plants using stem meristems, i.e., shoot tips and apical buds. Organogenesis is the in vitro propagation of plants from explants or callus where the organs (roots or shoots) are produced and then are manipulated to produce complete plants. Somatic embryogenesis is the in vitro propagation of plants, from single cells or small groups of vegetative cells, where the final stages of development produce embryolike structures that are capable of developing into intact plants. Although somatic embryogenesis appears to have the most promise for use with forest trees, each of the other methods also has its place in forest tree improvement work. The purpose of the discussion that follows is to illustrate how tissue culture techniques have and are being used in a hardwood tree improvement program in the Lake States. The hope is that in learning of these results, you will see ways you may be able to use similar approaches to solve problems associated with your tree improvement programs.

THE ASPEN GENETICS PROGRAM

The discovery of many rapid-growing, good-quality diploid and several triploid quaking aspen (Populus tremuloides Michx.) clones resulted in the establishment of an industry-sponsored aspen tree improvement program in 1955. The objectives of the program were to use the techniques of selection, hybridization and polyploidy to produce rapid-growing trees with improved wood properties. Many of the early ideas were those of Dr. Philip Joranson and were implemented by the first author and other researchers including Dr. Lawson Winton and Dr. J. P. van Buijtenen. During the 20 years that followed, more than 700 full-sib crosses were made, and the crosses included the hybridization of quaking aspen with P. alba, P. grandidentata, P. tremula, P. davidiana, and P. canescens. Crosses were made in the greenhouse using the cut branch technique of Wettstein[1]. The most often used procedure was to complete the crosses in February and March, produce 300-400 1-0 seedlings from each cross the following summer, and use these trees in one or two replicated field plantings. This crossing and field testing program resulted in the development of several types of crosses that appear to have potential for use by the paper industry. To date, the most useful crosses are those between P. tremuloides and P. tremula. Particularly promising have been a series of crosses using several P. tremuloides females and a tetraploid (4n) P. tremula male developed by H. Johnsson in Sweden[2]. As a result of this long-term research program, hybrid aspen seedling populations have been produced that at 20 years grow about twice as fast as native aspen, have 20-30% longer fiber length, and have about 8% higher wood density[3]. Associated with the described improved wood properties were improved paper properties (greater tearing and bursting strength and comparable tensile strength). Presently the forest management approach being suggested is to plant the triploid hybrid seedling populations at wide spacing (80-100 ft^2/tree) on medium to low quality northern hardwood sites, using conversion planting techniques. The prolific suckering ability of most aspen and aspen hybrids will then allow the management of the plantings using coppicing procedures for several rotations.

THE HYPDXYLON CANKER PROBLEM

Hypoxylon canker (*Hypoxylon mammatum* Wahl., Miller) is the most serious forest management problem influencing the use of aspen and aspen hybrids. Losses in the Lake States have been estimated to be 3% annually[4], an amount approximately equal to the annual harvest in 1983 of 280 million cubic feet[5].

One of the original objectives of the aspen genetics program was to select and breed for resistance to this serious canker disease. A modest amount of progress has been made by selecting and using parent trees that were free from hypoxylon. Progeny tests were evaluated at five-year intervals through age 25, and at present 20-year records exist for more than 60 full-sib aspen and hybrid aspen crosses. Typically, crosses with low resistance to hypoxylon will have infection levels of 46-68% at 20 years, whereas the best *tremuloides* x *tremuloides* crosses had 20-year infection rates of 0 to 18%*. Although progress has been reasonable, there is an urgent need to increase the number of highly resistant parent trees and to develop clones with a high degree of resistance for use in a planned clonal forestry program.

CHARACTERISTICS OF THE DISEASE

Hypoxylon canker is caused by the fungus *Hypoxylon mammatum* (Wahl., Miller). The wide geographic range of quaking aspen and the seriousness of the disease has resulted in the establishment of many research investigations into the nature of the disease and factors associated with spread of the disease. One of the most interesting was the discovery by Hubbes[6] of a diffusible substance produced by the fungus that elicited symptoms characteristic of the disease. The necrotic response to "mammatoxin" was later shown to be host-selective for species susceptible to hypoxylon canker and strongly suggests that the toxin is a determinant in the disease[7,8]. The necrotic response to mammatoxin resulted in the development of leaf bioassay by Bruck and Manion[9] in which the toxin was substituted for the pathogen in a procedure designed to identify disease-resistant clones.

Many important diseases of agronomic crops have been shown'to have host-selective toxins as determinants in pathogenesis[10]. Employing toxin in a tissue culture system has resulted in the isolation of toxin-resistant cell lines. In those cases where plants were regenerated from these cultures, toxin resistance often persisted[11,12]. Further testing of the plants with the pathogen resulted in equating toxin resistance with disease resistance. Considering the above success in agriculture and that mammatoxin appears to be a determinant in hypoxylon canker, the use of mammatoxin in tissue culture systems to screen for toxin resistant seedlings was a logical approach to attempt to produce cellular-level resistance to hypoxylon canker.

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* % is the number of individuals lost or presently infected with hypoxylon canker divided by the total number of field planted trees exposed to the disease.

TISSUE CULTURE SELECTION OF TOXIN RESISTANT ASPEN

During the summer of 1983 a student research program was initiated that had the purpose of isolating and propagating mammatoxin-resistant quaking aspen. Part of this Ph.D. research program was to develop a procedure that allowed the regeneration of plantlets from cotyledon explants. The procedure that resulted (Wann and Einspahr [13]) involved induction of multiple adventitous buds on cotyledon and hypocotyl explants by culturing the explants on MS medium containing 0.1 mg/L NAA and 1.0 mg/L BA. Explants producing multiple buds were then transferred to 1/2 MS (macro- and microelements) containing 0.3 mg/L BA for elongation. Root formation was achieved by transfer to 1/3 MS containing 0.1 mg/L IBA. Normally about 90 percent of the elongated shoots rooted, and transfer to soil was accomplished with little difficulty by maintaining high humidity conditions for one month after transfer.

The procedure used to screen and propagate mammatoxin-resistant quaking aspen is illustrated in Fig. 1. Small hypocotyl and cotyledon explants were placed on the "bud proliferation" medium in which mammatoxin was substituted for part of the water. Following four weeks on this medium, organogenesis was evaluated, and surviving explants were rescued from the toxin-containing medium and transferred to the toxin-free elongation medium. The surviving elongating shoots were rooted and transferred to soil. The resulting plantlets were then grown for 18 weeks and bioassayed using the previously cited bioassay method developed by Bruck and Manion [9] and described by Griffin, et al. [14] and Stermer, et al. [15]. The bioassay method consisted of removing three leaves from the 18-week-old aspen plantlets and placing the petiole of leaves in a small vial of water. Small holes were made in the leaves with a minutin insect pin, and a 3 drop of the properly diluted toxin was placed over the hole. Usually, three holes were made in each half of the leaf blade. Following incubation in a humidified chamber at 28°C for 48 hours, the response to the toxin was measured as lesion diameter to the nearest 0.5 mm .

An additional novel complementary study was run in which, for 120 seedlings, one cotyledon was removed and placed on the toxin containing screening medium. The original seedlings (minus one cotyledon) were grown for 18 weeks for use in leaf bioassay comparisons with those explants and resulting plants that survived the toxin screening and tissue culture propagation procedure. Figure 2 illustrates the procedure used. In this way, the bioassay response of a toxin-screened plantlet could be compared with the bioassay response of the donor plant.

The results of this toxin screening procedure and the leaf bioassay of the resulting plantlets turned out to be very interesting. When plants surviving the screening procedure were tested in the leaf puncture bioassay, this resistance was still maintained in the tissue-culture propagated ramets. For example, 5 clones comprising a total of 23 individuals were obtained from a full-sib cross, and all responded in a manner analogous to cottonwood, a species

*Additional details on producing the toxin and running the leaf bioassay are available in a paper by Wann and Einspahr [16].

that is resistant to hypoxylon canker and reacts negatively (lesion diameter < 1 mm) to the leaf bioassay. Equally interesting is that when the donor plants were compared with 22 clones of toxin-screened, cotyledon-derived plantlets (an ortet/ramets comparison), both the donor plants and corresponding toxin-derived plantlets tested resistant in the leaf bioassay. Figure 3 illustrates the results of in vitro toxin screening of two full-sib seedling populations.

As a result of these investigations it appears that mammatoxin can be used to rogue organ cultures of aspen seedlings for cellular level resistance to mammatoxin, and the plants propagated from these cultures retain this trait. The ability of an explant to survive and produce shoots on a toxin-containing medium is apparently inherent in the seedling from which it was derived, and not induced by the tissue culture system. Highly resistant individuals occurred to a significant extent in all crosses examined, indicating the resistance was of natural origin. This indicates that despite the current interest in genetic engineering (transformation, protoplast fusion, etc.), it appears the full genetic potential of many commercially important forest species is not now being fully utilized.

PLANNED EXPANSION OF THE ASPEN PROGRAM

Aspen is the most important pulpwood species in the Lake States Region. An attempt is being made to obtain an appropriate level of funding that will allow us to (1) determine the ability of the leaf puncture bioassay procedure to identify canker-resistant trees, (2) determine the heritability of mammatoxin resistance in quaking aspen, (3) verify the usefulness of tissue culture procedures in the production of a highly resistant parent tree for use in future breeding work, and (4) develop procedures for screening 15 to 25-year-old hybrid aspen populations for resistant individuals that can be used in a planned clonal forestry program.

The existence of appropriate parent trees and of 20-year field data on full-sib crosses will allow us to repeat crosses of interest and to compare the tissue culture seedling screening results and leaf bioassay data with the 20-year field results. The existence of parent trees and the ability to produce and evaluate progeny will also allow heritability estimates to be made. The existence of thousands of 15 to 25-year-old aspen hybrids in Northern Wisconsin plantings will serve as a source of rapid growing clones, a percentage of which may also turn out to be resistant to hypoxylon canker.

APPLICATION OF THE APPROACH TO OTHER HARDWOOD PROGRAMS

The tissue culture work by Wann described above represents the first instance where, for a commercially important forest tree species, a tissue culture system has been used to select resistant cultures and regenerate plants that maintain the resistance. This approach and similar procedures appear to be particularly appropriate for use with forest tree disease problems where the disease is of a toxin-determinate nature (Fusaria and Alternaria species, for example). This work also emphasizes the need for the development of several alternative tissue culture systems for the important U.S. tree species so that we can efficiently cope with future insect and disease problems.

LITERATURE CITED

1. Wettstein, W. V. 1933. Die Kreuzungsmethod and die Beschreibung von Fl Bastarden bei Populus. Zeitschrift fur ZUchtang, A. Pflanzenziichtung 18:579-626.

2. Einspahr, D. W. 1984. Production and utilization of triploid hybrid aspen. Iowa State J. of Research 58(4):401-409.

3. Einspahr, D. W.; Wyckoff, G. W. 1975. Aspen hybrids promise future source of Lake States fiber. Pulp Paper 49(12):118-119.

4. Marty, R. 1972. The economic impact of hypoxylon canker on the Lake States resource. U.S. Dept. of Uric. For. Serv. Gen. Tech. Rept. NC-1.

5. Blyth, J. E.; Smith, W. B. 1984. Pulpwood production in the Lake States by County. The Timber Producer, Sept., 1983:20-22.

6. Hubbes, M. 1964. New facts on host-parasite relationships in hypoxylon canker of aspen. Can. J. Bot. 42:1489-1494.

7. Schipper, A. L., Jr. 1975. Hypoxylon pathotoxin necessary to the infection by Hypoxylon mammatum. Proc. Am. Phytopath. Soc. 2:46-47.

8. Schipper, A. L., Jr. 1978. A Hypoxylon mammatum pathotoxin responsible for canker formation on quaking aspen. Phytopath. 68:866-872.

9. Bruck, R. I.; Manion, P. D. 1980. Mammatoxin assay of genetic and environmental predisposition of aspen by Hypoxylon mammatum. Plant Disease 64:306-308.

10. Scheffer, R. P.; Livingston, R. S. 1984. Host selective toxins and their role in plant diseases. Science 223:17-21.

11. Gegenbach, R. G.; Green, C. E.; Donovan, C. M. 1977. Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Nat. Acad. Sci. U.S.A. 74:5113-5117.

12. Hartman, C. L.; McCoy, T. J.; Knovs, T. R. 1984. Selection of alfalfa cell lines and regeneration of plants resistant to the toxin(s) produced by Fusarium oxysporum f.sp. Medicaginis. Plant Sci. Lett. 34:183-194.

13. Wann, S. R.; Einspahr, D. W. Reliable plantlet formation from seedling explants of Populus tremuloides (Michx.). Accepted Silvae Genetica. 1985.

14. Griffin, D. H.; Ehrenshaft, M.; Manion, P. D. 1980. Host-selective toxins of Hypoxylon mammatum for Populus tremuloides. Pages 209-214 in: Resistance Mechanisms in Poplar Diseases. Ed. by M. Ride. FAO International Poplar Commission, 21st Conference.

15. Stermer, B. A.; Scheffer, R. P.; Hart, J. H. 1984. Isolation of Toxins of Hypoxylon mammatum and demonstration of some toxin effects on selected clones of Populus tremuloides. Phytopath. 74:654-658.

16. Wann, S. R.; Einspahr, D. W. 1985. In Vitro isolation and propagation of mammatoxin-resistant quaking aspen. Submitted to Forest Sci.

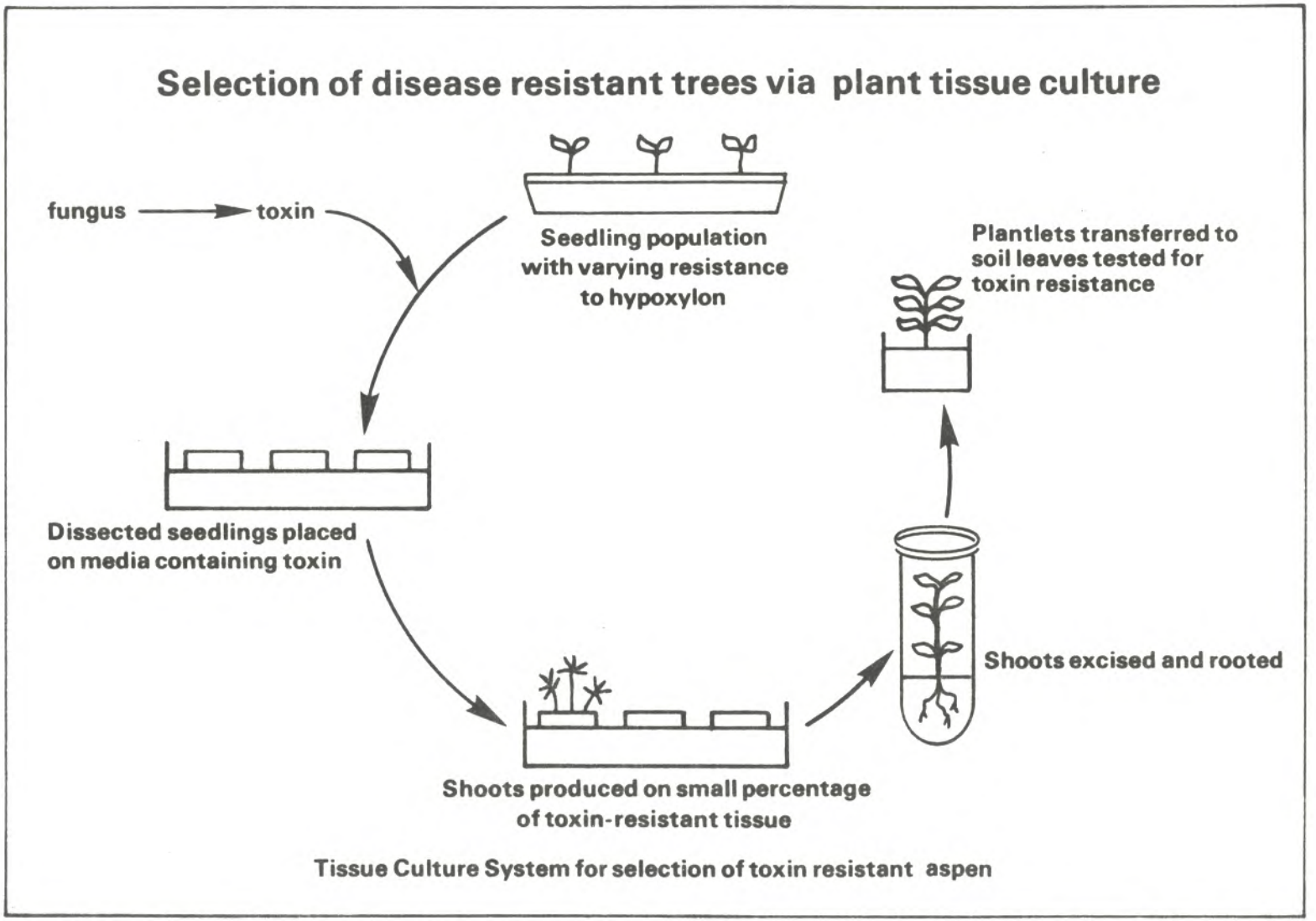

Figure 1. The method used for the selection of toxin resistant trees via plant tissue culture.

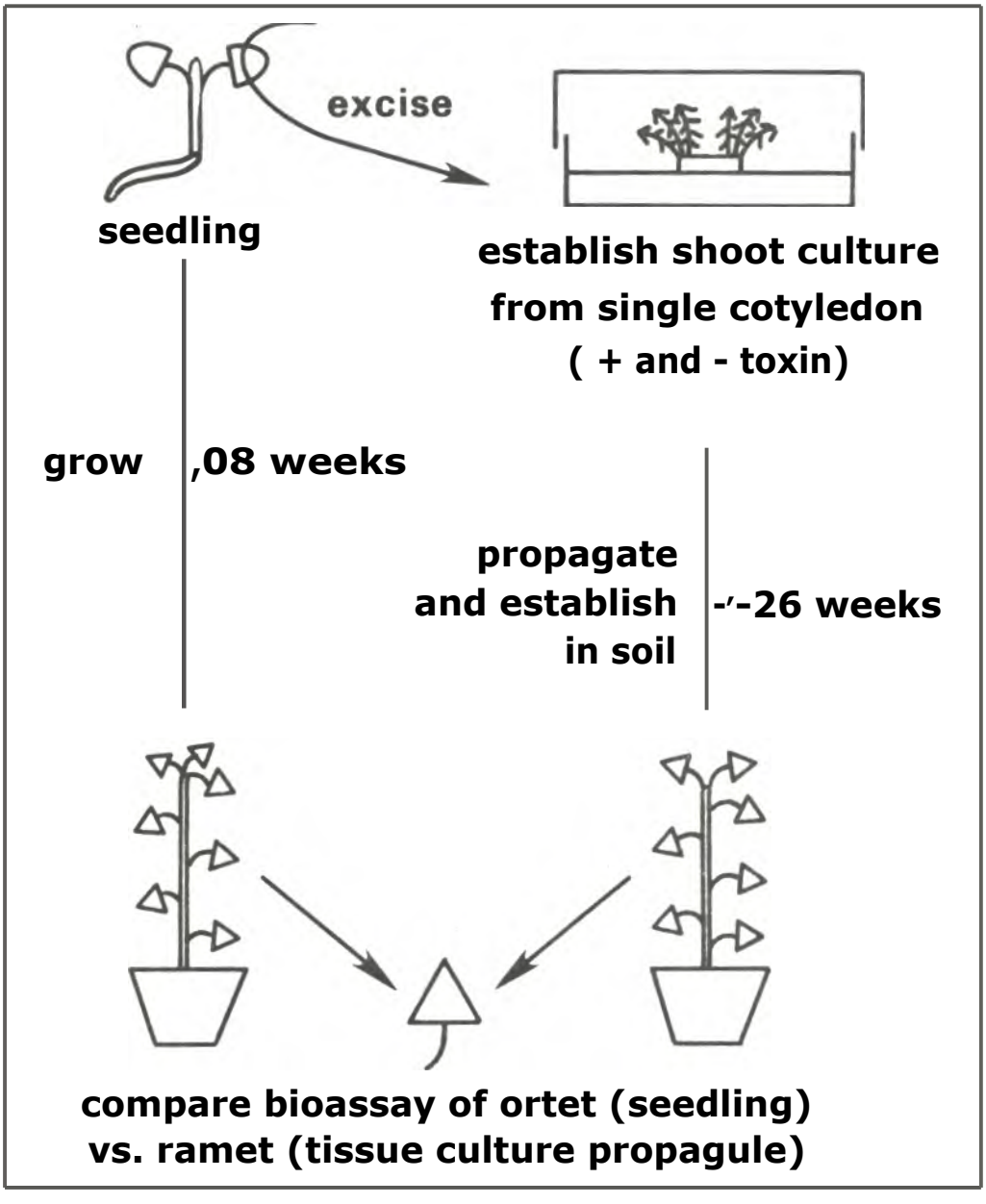

Figure 2. The methods used to propagate plantlets for the ortet vs. ramet bioassay comparison.

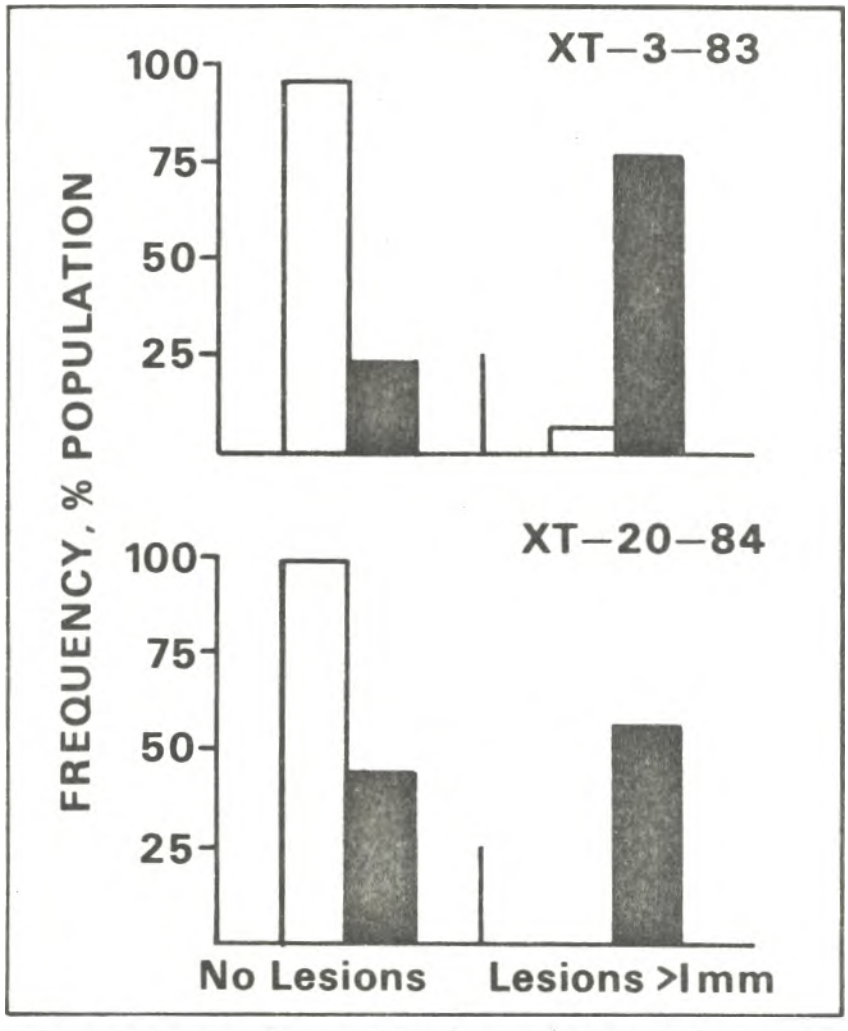

Figure 3. Illustrated by the open bars are percentages of toxin-resistant individuals (no lesions) vs. susceptible plants (lesions > 1 mm) from two aspen full-sib populations that were screened using the in vitro screening procedure. The closed bars indicate the inherent variation in toxin resistance in the original populations.