Somatic Embryogenesis As A Delivery System For Specialty Products With Reference To Resistant Sitka Spruce To The White Pine Weevil

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ABSTRACT

The recent discovery of resistant Sitka spruce (*Picea sitchensis* (Bong.) Carr) sources to the white pine weevil (*Pissodes strobi* (Peck)) in British Columbia has increased the hope of reintroducing this tree species as a regeneration option in high weevil hazard zones. In this paper, we summarize: 1- the efforts associated with the discovery of resistant sources from long-term provenance trials, 2- clonal screening to verify resistance, 3- the development of field and laboratory bio-assay methods for resistance determination, 4- preliminary research on the genetic mechanism of resistance, 5- role of somatic embryogenesis as a viable delivery system, and 6- initiatives related to the deployment of resistant stock.

Keywords: Sitka spruce, white pine weevil, breeding, somatic embryogenesis, resistance mechanisms, bio-assay, deployment.

INTRODUCTION

Destructive damage caused by the white pine weevil (*Pissodes strobi* (Peck)) to Sitka spruce (*Picea sitchensis* (Bong.) Carr) plantations has significantly reduced the planting program of this valuable coastal species. The annual planting has declined by more than 90% from a historical high of 10 million seedlings per annum. Consequently, less-desirable, species such as western hemlock and western red cedar are being planted as replacements. At present, planting of Sitka spruce is being restricted to low hazard sites (mid- and upper cost of British Columbia) or to those locations where the insect is not present (e.g., Queen Charlotte Islands). In response to these pressures on coastal reforestation, a breeding program for weevil-resistant Sitka spruce has been initiated during the past decade (King 1994). This breeding program is capitalizing on the large differences in weevil attack among Sitka spruce trees that are originated from a large provenance trial. In this paper, we present an overview on the discovery of resistance, work underway that is aimed to understanding the mechanism(s) of resistance, progress of breeding activities, methods for bulking-up resistant stock for regeneration, the development of field and laboratory bio-assay methods for resistance determination, and finally a conceptual idea of deployment methods of resistant stock.

Discovery Of Resistance To The White Pine Weevil

The IUFRO Sitka Spruce Provenance Trials

The IUFRO Sitka spruce provenance trials in British Columbia (BC) were planted at 14 locations from 1973-75 (Figure 1, Ying 1991). A total of 45 provenances were tested (Figure 2, Ying 1991). The 45 provenances (represented by bulk wind-pollinated seedlots) sampled the entire range of the species' natural distribution from Alaska (≈59° latitude) to southern Oregon coast (42°). Not all the 45 provenances were tested at all sites (for details see Ying 1991). Heavy weevil attack was observed on four out of the 14 test sites. These sites are: Nass, Kitimat, Head Bay and Sayward (Figure 1). Resistance sources were discovered on these four sites. The resistant sources originated from Haney (IUFRO #63, local #29, for cross-reference) and Big Qualicum (IUFRO #62, Local #03) as well as provenances from Sitka-white spruce hybridization zone (see Figure 2, Ying 1991).

Resistance evidence in provenance trials

Resistance was defined by either the cumulative number of attacks over all source trees over a specific period of time or by percent of trees with no attack over the same time frame. The weevil attack was noticeably evident in the provenance trial shortly after planting, thus exposing the plantations to high weevil pressure. Provenance variation in resistance/attack was evident in mid 1980s (Ying 1991). The Haney provenance showed the most resistant at the Sayward site with 0.6 cumulative number of attacks and 57% of trees with no attack as compared to 1.8 and 24% over the test site average, respectively (note that Haney provenance was only present at Sayward site). Similarly, the Big Qualicum source was the most resistant with 0.7 cumulative number of attacks and 60% of trees with no attack as compared to 1.7 and 16% over the three test site average, respectively (note that the Big Qualicum was planted on the Nass, Kitimat, and Head Bay only) (Ying 1991). Additionally, provenances from the hybridization zone (e.g., Kitwanga source) were also resistant. It is noteworthy that the susceptible provenances had a total of over 600 attacks in 15 years, and most of these attacks occurred in the last 5 years. This demonstrates that these provenance trials were subjected to very stringent screening of their ability to tolerate/resist weevil attack.

Repeatability of Resistance

A pilot study to test the repeatability of provenances' differences in weevil resistance was initiated in 1984 on a high weevil hazard site (Figure 1: Fair Harbour; Ying 1991). A total of 36 trees from eight different provenances were selected to represent the entire range of weevil attacks. The selected trees were grafted and planted in a clonal trial with each genotype represented by 16 ramets. The production of rooted cutting was not attempted due to the age of the ortets.

Only three grafts were attacked two-years after establishment (1986), but the attack level increased to 22% during 1988, and reached 57.3% in 1989 (Figure 7 in Ying 1991). After seven year, 66.7% of the grafts were attacked at least once, and the average number of attacks per graft was 1.03. These results were similar to that observed previously from the IUFRO provenance trial above. The Haney grafts showed the most resistance with only 14 of the 128 grafts (11%) were attacked. Similarly, grafts from the hybridization zone showed high level of resistance

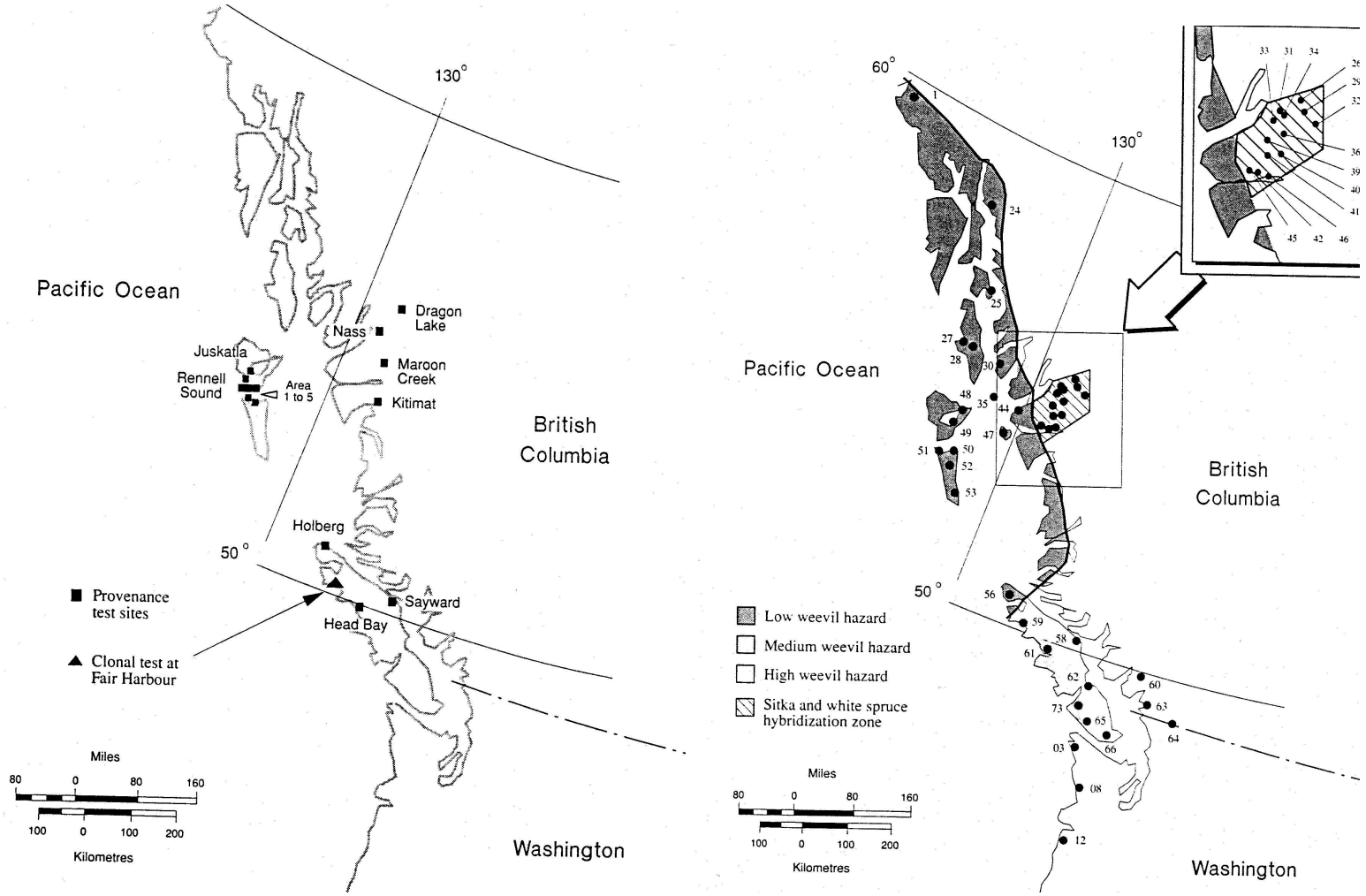

Figure 1. Location of Sitka spruce provenance trials and the clonal test for genetic resistance to the white pine weevil.

Figure 2. Locations of the population origins and test sites for Sitka spruce population trials in British Columbia.

(Ying 1991). On the other hand, grafts from the least resistant genotypes averaged 67% attack while one susceptible genotype had over 90% of attack. This trial confirmed the presence of resistance. Our ability to duplicate a wide array of genotypes, with various resistant levels, under high weevil hazed environment allowed us to verify the results obtained from the provenance trial. Thus providing us with confidence to start breeding for weevil resistance program.

Research On The Genetic Mechanism Of Resistance

The discovery of weevil resistance Sitka spruce genotypes indicated that the observed resistant sources originated from either the North Coastal region (known as the Sitka-white spruce hybridization zone) or from two pure Sitka spruce populations (Big Qualicum from East Vancouver Island and Haney from the Lower Fraser Valley) located within the high weevil hazard area that are associated with the dry coastal Douglas-fir biogeoclimatic zone of British Columbia (Figure 3). Under the assumption that the resistant is confined within the high weevil hazard-dry zone, King (1994) initiated a selection and breeding program that was designed to extensively sample this region. Within this zone, wind-pollinated cone collections were made along a North-South transect (Figure 3). A total of 67 wind-pollinated and 6 bulked seedlots were collected from British Columbia, Washington and Oregon. These samples also included seven wind-pollinated families from known susceptible sources, namely, the Queen Charlotte Islands (Figure 3). (A larger second series was also collected the following year, but is not reported here.) The sampled material provided seedlings for two test trials with 24 single-tree-plot replications. Ten out of the 24 replications were augmented with weevils (see testing spruce for resistance to the white pine weevil section below). Weevil augmentation was done to secure infestation and to speed the screening process. The trials were monitored for 4-5 years and weevil attack was recorded. The results indicated that the Sitka spruce resistance to white pine weevil is not confined to the high weevil hazard-dry coastal Douglas-fir zone but rather supported the presence of very limited resistant “hot spots” and that the predominant mode of genetic control of this resistance may be due to major gene action (King et al. 2001).

Somatic Embryogenesis

The ultimate goal of any tree improvement program is to maximize the genetic gain per unit time/area. Traditionally, seed orchards act as the factory where the genetic gain is being packaged and delivered to nurseries for the production of genetically improved seedlings needed for reforestation programs. However, in most cases, wind-pollinated seed orchards have fallen short of expectations and several organization/countries adopted the path of making elite crosses followed by vegetative propagation for the production of material with high genetic gain. This approach made family forestry feasible, and the time and expense required for serial propagation and the rooted cutting production were certainly justified by the additional gain. It is also important to point out that in spite of the additional gain attained by family forestry, this gain represents only the average of the parents used in the elite crosses. Thus, foresters are deprived from exploiting the within family selection that is associated with sexual reproduction. In simple terms, within family variation could not be assessed or evaluated by family forestry due to the fact that the time required for within family comparison will affect the age and hence the juvenility of the donor plants. Somatic Embryogenesis (SE), on the other hand, provides a tool that overcomes the limitations of both the classical delivery systems through seed orchards and family forestry and providing the most viable option for practicing clonal forestry.

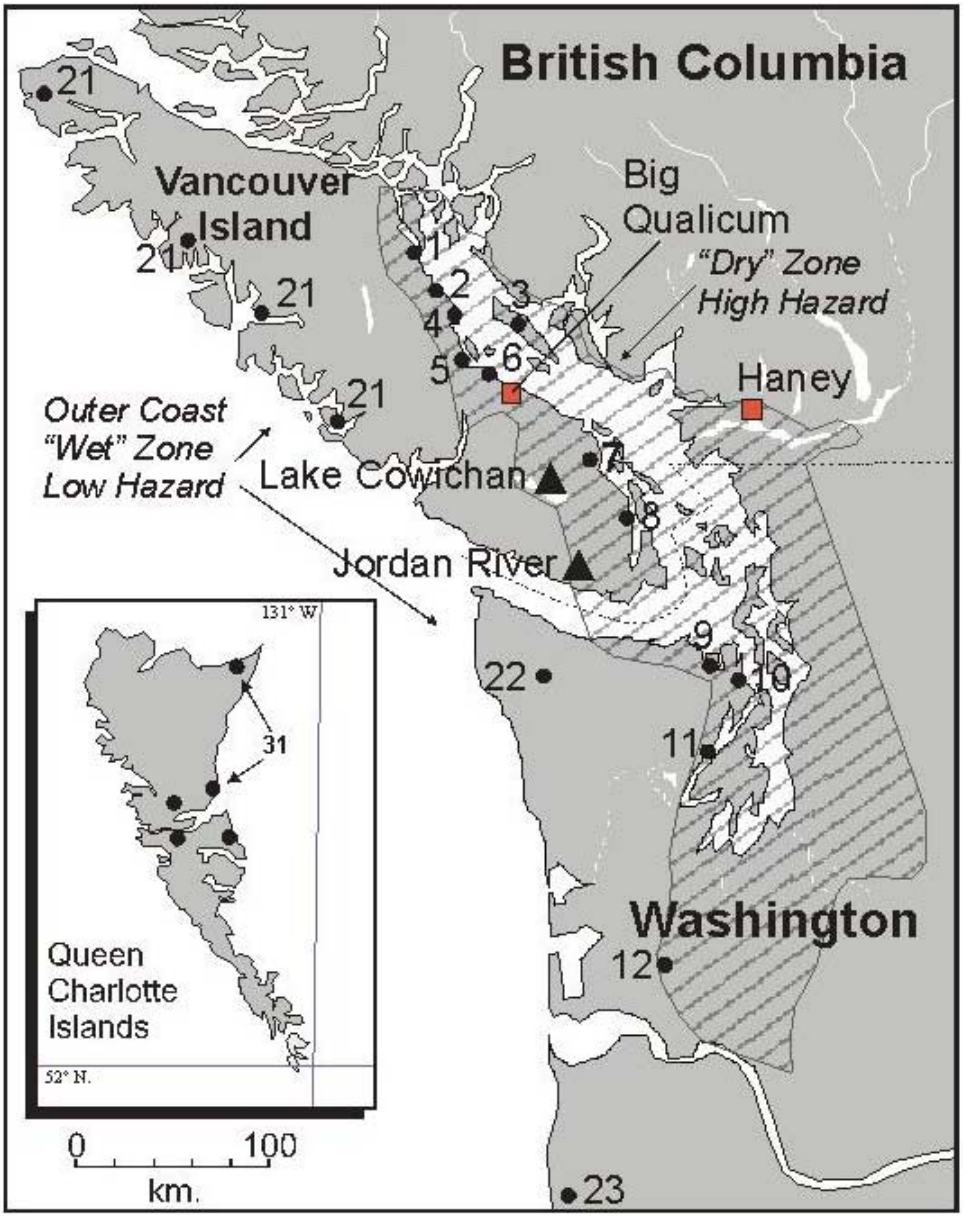

Figure 3. Original resistant sources from the IUFRO provenance trials (big Qualicum and Haney) are represented by solid squares and location of the sample location from the high weevil hazard-dry coastal Douglas-fir zone represented by solid circles.

The Technology

SE was independently reported in conifers by Chalupa (1985), Hakman et. al. (1985) and Nagmani and Bonga (1985). The SE process of CellFor Inc. includes several steps (Figure 4). These are induction, cryopreservation, liquid culture multiplication, somatic embryo maturation, desiccation, germination and transplanting of embryos to seedling containers for subsequent growth in the nursery. Most of the information pertaining to induction is published and available in the public domain (see the above-mentioned references). However, several of the following steps are proprietary and many of them are either patented or are in the process of being patented. These are described, as follows:

1- **Induction:** involves placing mature or immature embryos on a sterile culture medium and incubation to allow somatic tissues to develop. The tissue developed from each individual mature or immature embryo consists of a mass of immature embryos with one single genetic identity that is capable of continuous proliferation.
2- **Cryopreservation:** is the storage of developed somatic tissue produced during the induction in a frozen condition in liquid nitrogen. This step is important for maintaining the somatic tissue for a long period of time. It could be considered as a clone bank or gene conservation bank of unique genotypes, and provides tree breeders and foresters the time required to conduct clonal testing (clonal testing will be explained below).
3- **Liquid culture multiplication:** represents the true cloning step in the process (commonly known as bulking up). During this step, the induced tissues are exponentially multiplied. The multiplied tissues are undifferentiated and are used for the actual production of embryos.
4- **Somatic embryo maturation:** treats and separates the undifferentiated tissues produced during the cloning step into well-differentiated embryos. CellFor uses a proprietary method using bioreactors to produce these mature, synchronized well-differentiated embryos in high numbers (tens of thousands to millions).
5- **Desiccation:** this step is unique to CellFor's proprietary technology. It mimics natural seed development and provides a technology that allows CellFor to produce embryos at any time of the year, without the restrictions imposed by the production of embryos with high moisture content that can not be stored. This makes it possible to produce millions of embryos that are ready for planting during the narrow biological windows of seedlings production.
6- **Sowing and Germination:** are similar in principle to the sowing and germination of zygotic seed in a greenhouse environment. However, there are special challenges because the somatic embryo lacks a megagametophyte and seed coat. Accordingly this step has been the subject of intensive research and development activities. Various research groups are considering different approaches such as the production of synthetic seeds (i.e., encapsulation) or the used off naked embryos. CellFor's proprietary technology uses the latter methods in which treated embryos are mechanically sown into mini-plugs that can be transplanted into either container or bare-root nurseries for seedling production.

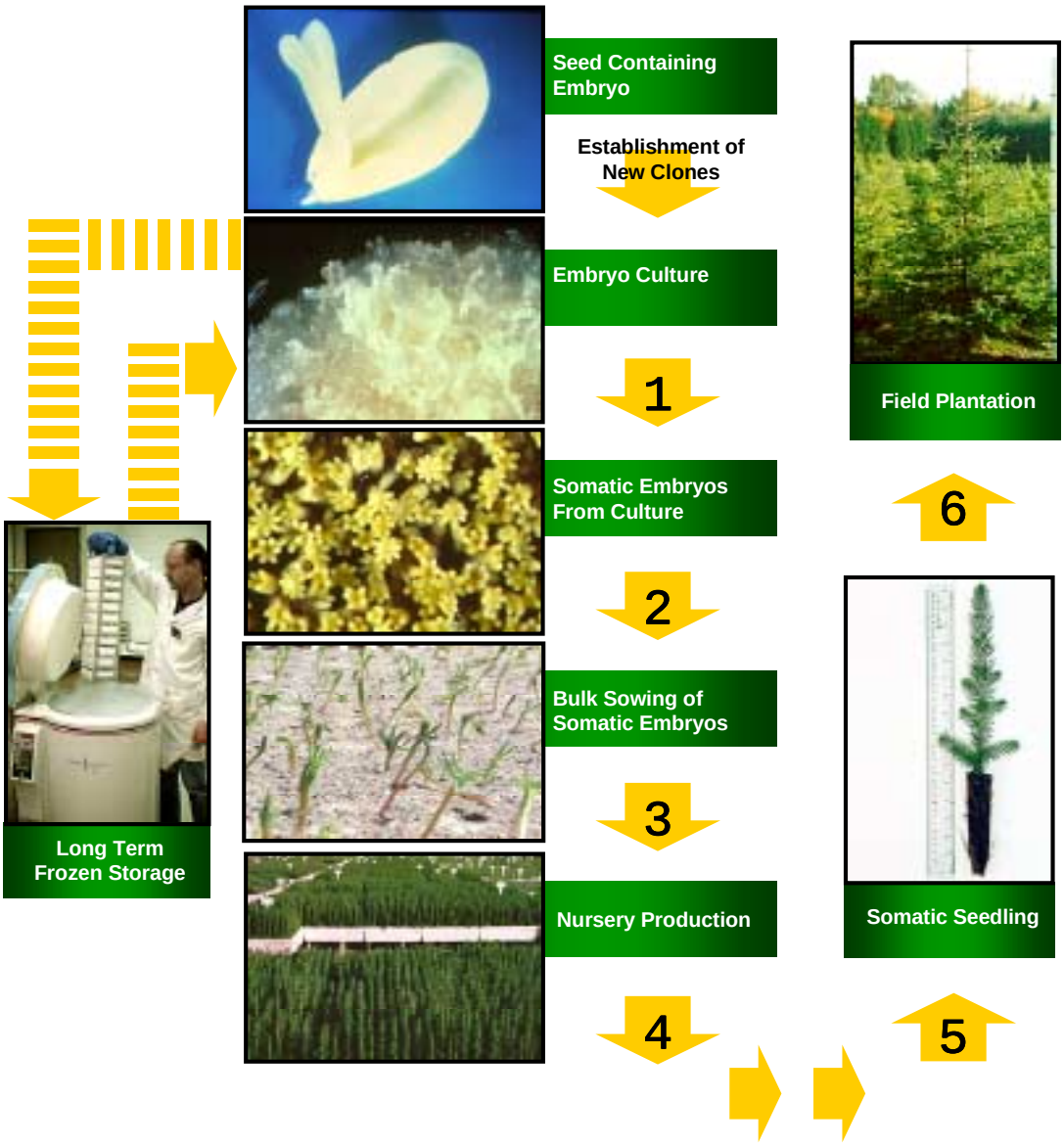

Figure 4. Diagram showing CellFor's somatic embryogenesis delivery systems.

Importance of clonal testing

Genetic segregation and recombination among genes are fundamental to sexual reproduction. They represent the mechanism responsible for the genetic similarities and differences among individuals within a family. Thus, when controlled crosses are conducted among elite parents in a tree improvement program, the offspring are expected to exhibit variation. When clonal forestry is practiced, the cloning process acts as an amplifier. The amplification power can be harnessed and exploited to provide the foresters with the maximum genetic gain that can be attained. Thus, before large-scale production of a particular clone is commenced, it is recommended that clonal tests be conducted. When a cross is made between elite parents,

several seeds are produced. The SE technology produces several clones (a clone is a representative of a single seed) for each cross. Results from the clonal testing phase will identify the desirable clones. These clones represent the ones that go through the amplification process and thousands or 100s of thousands copies are produced for reforestation. It is important to note that the number of crosses and the number of clones within each cross are important factors to consider during the implementation of clonal forestry program, so the level and distribution of genetic variation within plantations are optimized (see deployment section below).

Where and when SE should be implemented?

It is important to state that SE should be used to augment the classical tree improvement delivery systems and not to be viewed as a replacement. High genetic gain clones should be considered for high productivity sites, thus allowing the forester to get more wood from less land. Clones with high resistance level to pests, such as white pine weevil, can effectively be produced through SE as a specialty product. These specialty products can not be produced through sexual reproduction, or even rooted cuttings after sexual reproduction, due to the limitations explained above. Additionally, SE could be utilized in the testing phase of breeding programs in order to provide the breeder with progeny with high genetic uniformity.

Cost vs. value

The common dogma is that the development costs of SE are high and thus its place in forestry is very limited. This view reflects the philosophical difference between the cost oriented forester and the value-oriented executive. Any new technology should be considered based on the value that it will generate. Economic analyses that consider the costs of SE vs. conventional forestry methods have proven that the value of SE far exceeds conventional methods when it is being applied to good and high quality sites (see El-Kassaby and Moss, this issue). Therefore, the objective way of evaluation is to consider the value that can be attained from SE net of the costs.

Testing Spruce For Resistance To The White Pine Weevil

Testing for resistance to insects and diseases is a complex issue. The reason being that plant resistance depends on a number of interacting traits, which vary with the age of the plant and are modified by the environment. Panda and Kush (1995) summarized this complexity stating that plants rely on a combination of defense mechanisms to fend off herbivores. A common defense strategy is providing the insect an improper nutrition, for example, by being in the wrong phenological state at the time of feeding. ***Constitutive defenses*** are structures or defense systems that occur regardless of the presence of the attacker. Examples of constitutive defenses include plant trichomes, thorns and latex and resin canals. ***Inducible defenses*** are those that are activated in response to herbivore attack. Examples of inducible defenses are the mobilization of defensive chemicals to the site of wounding, and the production of traumatic resin in conifers in response to insect and fungal attack (see below).

The methods to use in screening for resistance depend on whether the objective is mass screening for accelerated breeding programs or the identification of specific resistance mechanisms. Some of the methods used to evaluate resistance to white pine weevil are discussed below.

Field-testing

This involves the planting of candidate genotypes in replicated trials. Genotypes are discriminated as resistant or susceptible by exposure to natural (Alfaro and Ying 1990) or artificial infestations (King and Alfaro 2001). Using artificial infestations, the Canadian Forest Service completed the screening of over 29,000 Sitka spruce genotypes for resistance to weevil (Alfaro 2000). Use of artificial infestations is recommended since screening is faster and more reliable than depending on natural infestations to develop. Artificial infestations are initiated once plants reach suitable height for attack (3-5 years in coastal BC). Results are obtained within one or two years after infestation. This method is inexpensive and effective, however it does not provide clear indications of the resistance mechanisms involved.

Cage testing

Testing genotypes in cages, which are inoculated with the insects, provide the researcher with more information on the nature of the resistance. However, this method is laborious and therefore suited for detailed study of specific genotypes. Confined insects do not display all behaviours associated with the encounter of a resistant plant, interfering with dispersal movements. The arrangement of the plants in the cage is also important. Genotypes exposed to weevils in cages, as single genotypes, provide a no-choice situation for the insects, which must feed or starve, oviposit or re-absorb its eggs. Choice situations involve the mixing of genotypes within cages, giving the insects freedom to move and to select plants to attack according to their own preferences. In BC we have used both methods, thereby providing information on the resistance levels of specific genotypes.

Often clones that are highly resistant in the field could be readily attacked in cage situations. Testing the highly resistant Sitka spruce clones from the Haney provenance, Alfaro (1996, and unpublished observations) found that this provenance, which has shown field resistance to the white pine weevil (Alfaro and Ying, 1990; Ying, 1991), could be colonized in cage experiments. We attributed this to the fact that confined weevils could not disperse to more suitable hosts.

Cage experiments allow the study of insect behaviour. Alfaro (1996) observed the movements of weevils in cages containing the highly resistant Haney clones mixed with susceptible stock (a choice situation). Movement away from the resistant clones and settling on susceptible genotypes was indicative of repellency.

Klimazewsky et al. (2000) used cage experiments to test Sitka spruce genotypes of the Big Qualicum provenance, another resistant source, in a choice situation. They concluded that toxicity to larvae, possibly by resin, was a major cause of resistance in these genotypes.

Testing for specific resistance mechanisms

Variation in several traits of the host tree has been associated with the resistance to white pine weevil attack. These include variation in the chemical composition of feeding stimulants and deterrents (Alfaro et al., 1980), differences in resin canal density (Alfaro et al., 1997; Tomlin and Borden, 1994, 1996) and production of traumatic resin (Alfaro, 1995; Tomlin et al., 1998). Differences in the physical and chemical properties of the resin are also thought to play a role in resistance. These mechanisms often occur simultaneously, each one playing some role, but the relative importance of each defense system varies in different genotypes. Resistance mechanisms influence the physiology and behaviour of *P. strobi*. Weevils normally reject resistant trees, but if forced to feed on them or on unsuitable host tissues, they can sustain

ovarian regression and possibly other physiological degradation (Gara and Wood, 1989; Leal et al., 1997; Sahota et al., 1994; Trudel et al., 1998).

Plant phenology plays an important role in trophic relationships within an ecosystem. Early or late bud-burst or rate of shoot growth affect the quality and quantity of food available for herbivores at specific times (Quiring, 1992; Langvatn et al., 1996) directly affecting herbivore population levels. Differences in phenological development of allopatric plant populations may be correlated with seasonal variation in plant defenses, such as synthesis of resin and other defensive chemicals (Muzika et al., 1993). Testing for phenology defenses involves comparative study of the tree phenology (stages of bud-break and shoot elongation) and the timing of the various insect stages: oviposition, larval maturation, pupation and emergence. Working with Sitka spruce, Hulme (1995) and Alfaro et al. (2000) found that resistant Sitka spruce families tended to initiate growth earlier than susceptible families. However, considerable family to family variation existed. Hulme (1995) also demonstrated that if the synchrony between the phenology of the Sitka spruce genotypes and the phenology of *P. strobi* was altered, the white pine weevil could successfully attack the resistant genotypes.

Testing for constitutive defenses

In BC extensive studies were conducted on bark resin canal density in Sitka and white spruce. These studies indicate that some resistant tree populations rely heavily on resin canal systems for defense but not others. The resistance of spruce to weevil has been correlated to bark resin canal density in several studies (Alfaro et al 1997, and Alfaro et al 2000, Grau et al 2001, Tomlin and Borden 1994). In Sitka spruce, where resistance is found mainly in two populations, Haney and Big Qualicum, we fond that, resin canals are an important defense system for Haney (Tomlin et al. 1994, Grau et al, 2001) but not so much for the Big Qualicum populations. Resin canal densities of resistant Big Qualicum are only marginally higher than susceptible genotypes (Tomlin et al 1994, Brescia 2000). Testing resin canal densities requires standardization in the season. Because of shoot radial growth, the density of resin canals diminishes through the season (Brescia 2000). In addition to resin canal density, we have studied the presence of sclereid cells in the shoot phloem, the food for the weevil adult and larvae. These thick-walled cells are significantly more numerous in some resistant, relative to susceptible genotypes (Grau et al 2001).

Testing for inducible defenses

In 1995 it was discovered that spruce trees produce rings of traumatic resin canals in the shoot xylem, in response to weevil attack (Alfaro 1995) (Figure 5). This discovery opened new avenues for developing methods for artificially inducing this response and correlating it with resistance. Tomlin et al (1998), Brescia (2000) and O'Neill et al (2000) used artificial wounding to distinguish genotypes with different ability to produce traumatic resin. Resistant genotypes tended to produce a quicker and stronger level of traumatic resin than susceptible plants. Nault and Alfaro (2001) and Plant (A. Plant, Pers. Comm. Dept. Biology, Simon Fraser University, Burnaby, BC, Canada) found that artificially wounded plants initiated terpene synthesis quickly after wounding.

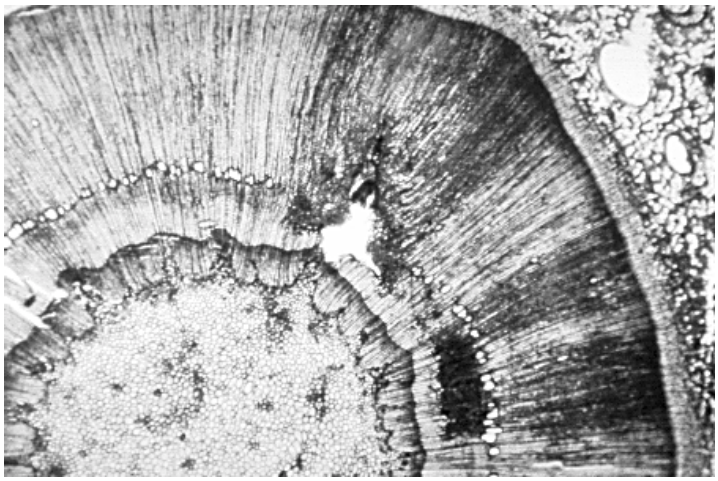

Figure 5. Cross section of an artificially wounded Sitka spruce shoot. Trees respond by creating rings of traumatic resin canals which remain embedded in the xylem.

The use of molecular markers

Reproductive maturation and oviposition of the *P. strobi* are inhibited by resistant Sitka spruce genotypes. Vitellogenin is an egg-yolk protein precursor, which is necessary for the maturation of eggs. A fragment of the vitellogenin gene from the white spruce terminal weevil was cloned and the DNA sequence of this fragment has high identity to vitellogenin sequences from other insects. It hybridizes on Northern blots to a single 6.0 kb mRNA that is expressed only in females, and only after they have started reproductive development. Vitellogenin gene expression is induced by treatment with juvenile hormone, and is differentially regulated in insects feeding on resistant or susceptible trees. It was observed that the expression of the vitellogenin gene is greater in weevils feeding on susceptible trees than in weevils feeding on resistant trees (Leal *et al.*, 1997).

It was also observed that the levels of ovarian growth and transcription of the vitellogenin gene are reduced in weevils feeding on the severed leaders from resistant trees relative to those feeding on severed leaders from susceptible trees. A force-feeding method was developed to deliver extracts from the bark of leaders into the alimentary canal of the weevils. Weevils given one dose of the aqueous extract from resistant leaders, followed by feeding on sections of laterals from susceptible trees, have exhibited 60% inhibition of oocyte growth and 48% inhibition of transcription of the vitellogenin gene relative to insects given the extract from susceptible leaders (see Figure 6 below). These results indicate that the effects of resistance do not require an intact tree, and experiments using extracts show that the observed effects result from a post-ingestive effect of the extract (Sahota *et al.*, 2001). The use of the vitellogenin gene as a probe may provide a sensitive bioassay for identifying resistance factors.

Deployment Resistant Stock

The question of how to best deploy selected clones (genotypes) has been one of the most interesting challenges for forest tree breeders and managers, over the past decade or two. The general issues of concern are well known, but little work, experimental and/or theoretical, is

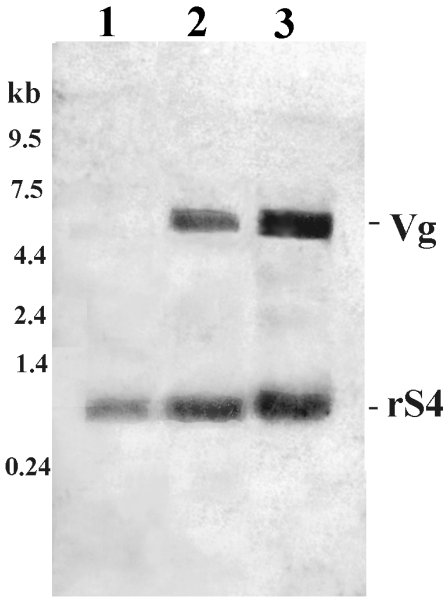

Figure 6. Northern blot showing expression of the vitellogenin gene (Vg) and small ribosomal subunit protein 4 (rS4) in female weevils that were force-fed with water soluble extracts of bark from resistant and susceptible leaders. Lane 1 (control), mRNA from a single female weevil (day 0), lanes 2 (resistant) and 3 (susceptible), mRNA from two female weevils (day 4).

available to provide support and clear conclusions. The work of Libby (1982) moved the question forward a great deal, and others have followed with further refinements (e.g., Bishir and Roberds 1995). Although, this work has formed the basis for choosing appropriate numbers of genotypes, there are still outstanding questions of how to best deploy them, in pure large clonal blocks, small clonal block mosaics, or in random mixtures, for currently known and unknown (i.e., risks to pest and disease) traits. While operational foresters are moving towards more genetically uniform block plantations (i.e., clonal blocks), there are several remaining unanswered questions related to risk. These questions are over and above the main question of the required minimum number of clones. At present, several risk related questions are being examined. These are concerned with the selection of superior genotypes for improved growth as well as pest and disease resistance.

The current research approaches to address these questions rely primarily on computer simulations and modeling. Specifically the Tree and Stand Simulation Models developed in British Columbia (Mitchell 1986) coupled with pest and disease population dynamic models (model details are reported by Yanchuk and Bishir in the North American Forest Quantitative Genetics meeting, Athens 2001). For instance, one of the scenarios examines clonal selection (both for a sets of 2, 6, 18 and 30 random or fixed clones), for three resistance mechanisms that approximate the current resistance systems in spruces. When sets of 2, 6, 18 and 30 clones are selected at random from a population, the results always indicate that random mixtures represent the superior deployment approach (Figure 7).

These results are preliminary, and only consider one of many scenarios that must be factored into a generalized deployment scheme, but at this point it seems that random mixtures of

genotypes are superior under the assumptions considered. These results will help further refine the deployment guidelines adopted in British Columbia, which currently require an effective population size of 10 and random mixtures.

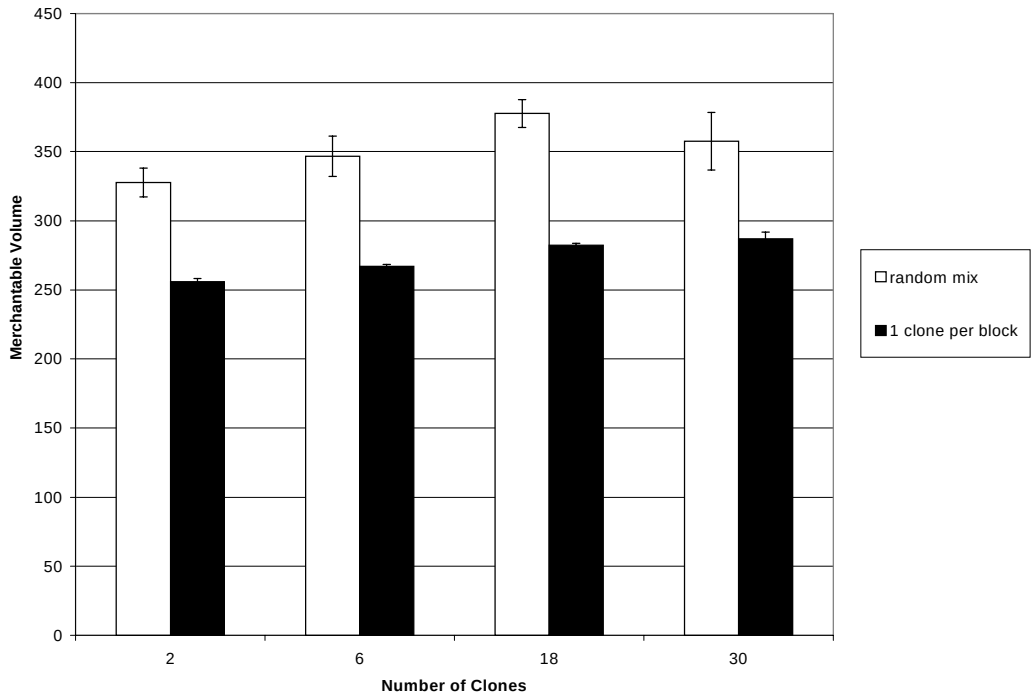

Figure 7. Merchantable volume of Sitka spruce produced when sets of 2, 6, 18 and 30 clones are selected at random from a population assuming that the weevil population is randomly distributed.

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