# Creating a somatic embryogenic system to study resistance traits to the white pine weevil (*Pissodes strobi* Peck.) in Sitka spruce (*Picea sitchensis* (Bong.) Carr).

by

Natalie Annastasia Prior
B.Sc., University of Victoria, 2008

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of the Requirements for the Degree of

MASTER OF SCIENCE

in the Department of Biology

# Supervisory Committee

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# Abstract

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A somatic embryogenic system was created using material from the British Columbia Ministry of Forests and Range's Sitka Spruce (*Picea sitchensis* (Bong.) Carr) breeding program for resistance to the white pine weevil (*Pissodes strobi* Peck.). The goal was to provide a system that could aid in understanding the phenotypic and genotypic variation that exists in these traits. Embryogenic lines were derived from controlled crosses of parental genotypes previously ranked for the abundance of three physical bark traits: sclereid cells, constitutive resin canals and traumatic resin canals. The number of filled seeds per cone from controlled pollinations was low, with a mean of 9.4 ± 6.8 (mean ± SD), compared to open-pollinated material, which had greater than 40 seeds per cone. The mean induction rate (to embryogenic cultures) was 7 %, ranging from 0 % to 56 % by cross. Of 135 genotypes, 88.1 % produced mature embryos. The number of embryos produced varied by culture. Nearly all (44 of 45) genotypes germinated, with a mean germination rate of 80 %. The overall conversion rate of somatic embryos to plants was 5.5 %. A novel method of cryopreservation that used a temperature pretreatment but did not require dimethyl sulfoxide was tested. Embryogenic cultures were recovered from 31 % of genotypes (n = 112). Genotypic and phenotypic variation were observed during each stage of the somatic embryogenic process. This project demonstrated that somatic embryogenesis and cryopreservation can be used to create a system to study phenotypic and genotypic variation in Sitka spruce.

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| | |
|---|---|
| 2,4 – D | 2,4 – Dichlorophenoxyacetic acid |
| ABA | Abscisic acid |
| AFLP | Amplified fragment length polymorphism |
| BA | Benzylaminopurine |
| DMSO | Dimethyl sulfoxide |
| PEG | Polyethylene glycol |
| PGR | Plant growth regulator |
| PVC | Polyvinyl chloride |
| QTL | Quantitative trait loci |
| RAPD | Random amplified polymorphic DNA |
| RFLP | Restriction fragment length polymorphism |
| SD | Standard deviation |
| SE | Somatic embryogenesis |
| SSR | Simple sequence repeat |

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# Chapter 1: Introduction

Sitka spruce (*Picea sitchensis* (Bong.) Carr) is a majestic member of the forests along the west coast of North America. It is prized not only for its beauty, but its role in coastal ecosystems and in forestry. Sitka spruce is valued for its strong but light timber (King and Alfaro 2009). However, in British Columbia, Sitka spruce is under attack. Young plantations are difficult to establish due to their susceptibility to the white pine weevil (*Pissodes strobi* Peck.) (King and Alfaro 2009). This has resulted in a drastic reduction in the use of Sitka spruce for reforestation. This reduction, deemed unacceptable, has led to great efforts in understanding and resolving this problem (Alfaro et al. 2008).

Sitka spruce is native to the west coast of North America (Xu et al. 2000). It has a long, narrow range that stretches over 22 degrees of latitude from Alaska to California, and inland following river valleys, where it can hybridize with other spruce species such as white spruce (*Picea glauca*) (Xu et al. 2000). It has one of the highest growth rates of all conifer species planted in Canada (O'Neill et al. 2002). It also has desirable wood characteristics (Alfaro et al. 2008) resulting in high log prices (O'Neill et al. 2002). Historically, Sitka spruce had high planting rates of up to 10 million trees per year. This has since been reduced to less than 1 million trees per year because of damage to young stands by the white pine weevil (King and Alfaro 2009 and references therein). By 2002, Sitka spruce was only used on about 2 % of reforestation sites (O'Neill et al. 2002).

In the 1930s, it was first noticed that some trees seemed to show a strong resistance to attack by the white pine weevil. This occurred at the Green Timbers

plantation in Surrey, British Columbia, where five trees survived a severe infestation that killed the rest of a ten hectare plantation (King and Alfaro 2009). Since then, the Sitka spruce and white pine weevil system has been extensively studied through provenance and progeny trials, and accompanying anatomical and entomological studies. These studies have provided hope that a long-lasting genetic resistance may be found for Sitka spruce (King and Alfaro 2009).

**Weevils and Resistance**

The white pine weevil is native to North America. It has a very large range that stretches across the continent, from the Yukon and Northwest Territories, to central Colorado and northern Georgia (Lewis et al. 2000). The white pine weevil is a pest to many coniferous species, including: Sitka spruce, Engelmann spruce (*Picea engelmannii)*, white spruce, eastern white pine (*Pinus strobus*), jack pine (*Pinus banksiana*), Norway spruce (*Picea abies*), and hybrids such as white x Engelmann spruce and white x Sitka spruce (Humble et al. 1994 in O'Neill et al. 2002). It is considered the most serious pest affecting regeneration of Sitka spruce (Tomlin et al. 1996).

Adult white pine weevils overwinter in the duff of the forest floor (Silver 1968). In the spring, the weevils move up young trees to the terminal leader. There they mate. Weevils puncture the bark of the terminal leader as they feed, and lay eggs in the resulting cavities. The punctures are sealed with fecal plugs. When the larvae hatch, they mine down the phloem of the leader. As they move down, their feeding galleries merge, creating a "feeding ring". Destruction of the leader by feeding on the phloem effectively girdles the tree. The weevils then pupate in chambers created in the xylem. From July through September, new adult weevils emerge and begin to feed. When

temperature and photoperiod decrease, the weevils return to the forest floor to overwinter (Silver 1968).

Damage to the terminal leader by the white pine weevil leads to stem deformities including crooks or forks. This damage reduces volume and height growth, and in some cases causes trees to be unsellable as timber (Alfaro 1996a, b). Stands suffering from severe infestations can lose up to 40 % of their volume (Alfaro 1992, 1994 in Alfaro 1996b). It is interesting to note that white pine weevil infestations are not common in natural stands (Alfaro 1996b). Here, infestations are only found near the forest edge or in gaps created by fire or wind. Natural stands tend to be cooler and shadier, provide less feeding material and fewer oviposition sites than plantations. They also have more complex food webs that include parisitoids of the white pine weevil (Alfaro 1996b). In regenerated stands, conditions are different. They provide abundant food and oviposition sites. They also accumulate the heat sums necessary for weevil development (Alfaro 1996b). This leads to outbreaks of weevils in stands beginning at age five years (Alfaro and Omule 1990).

Resistance mechanisms against the white pine weevil have been studied extensively. In many conifer species, the resin canal system plays an important role in defense. When resin canals are punctured, resin flows into the wounds, cleansing and sealing them (Berryman 1972). Resin may also function to deter or kill attacking insects, such as the weevil. Resin flows into the feeding and oviposition chambers, thereby killing or expelling weevils and their larvae (Alfaro et al. 1997).

Resin canals exist as part of the constitutive defense system in conifers, meaning they are part of the regular development of the tree and are always present in case of

attack (Alfaro 1995). Constitutive resin canals develop in the cortex of the stems (Alfaro et al. 1997), but also extend into the needles of the tree (Weng and Jackson 2000). It has been shown for Sitka spruce (Tomlin and Borden 1994), white spruce (Alfaro et al. 1997) and interior spruce (Alfaro et al. 2004) that increased density of constitutive resin canals may partially account for increased resistance to attack by the white pine weevil. Tomlin and Borden (1994) showed that sources of Sitka spruce that were resistant had resin ducts that were twice the diameter as those of susceptible trees. When viewed by transverse section, resistant sources had up to 28 resin ducts per centimeter, whereas susceptible sources had as few as 1 resin duct per centimeter.

A second type of resin duct system is also known. These are traumatic resin ducts (Alfaro 1995). They are induced only after a stimulus, such as an attack by the weevil. In white spruce, Alfaro (1995) found that after feeding or oviposition by the white pine weevil, the cambium began producing traumatic resin ducts in a ring in the xylem. Grau (2006) also reported traumatic resin duct formation in Sitka spruce.

Other aspects of weevil-resistance mechanisms have been explored. Entomologists investigated feeding and oviposition preferences of the white pine weevil, and found differences between previously identified resistant and susceptible trees (Tomlin and Borden 1996). Other studies have looked into possible chemical explanations for resistance. The role of volatile compounds that may attract or deter the weevil has been studied. Some examples include foliar and cortical monoterpenes (Harris and Borden 1983), and cortical resin acids (Tomlin et al. 1996). However, the results were not completely clear. Hulme (1995) suggested that some trees may appear resistant to white pine weevil attack because of differences in phenology related to their

origin, and that this causes a disconnect between the time of oviposition and the stage of development of the tree. Budburst phenology has also been linked to resistance. Alfaro et al. (2000) found that bud development occurred at a faster rate in resistant trees, and that resistant trees had a lower heat sum requirement than susceptible trees. Other physical characteristics have also been considered. For example, thin bark in combination with high number of resin canals may make trees less desirable to weevils (Tomlin and Borden 1997). Finally, increased density of sclereid cells in the bark, which make the tree inedible to weevils, has been suggested as another possible mechanism of resistance (Alfaro et al. 2002).

An interesting point is that many of these studies used trees that were known to be either resistant or susceptible, but in some cases it was found that although there was a general trend in the characteristic being examined, an exceptional case would occur where a highly resistant tree would not have the resistance characteristic. For example, Tomlin and Borden (1994) found that not all resistant trees had exceptionally high numbers of resin canals, and Tomlin and Borden (1996) found in feeding assays that not all resistant trees completely deterred weevils from feeding. This suggests that trees may rely on a variety of mechanisms for their resistance rather than depending on only one form of resistance at a time (Tomlin et al. 1997).

**Tree Breeding for Weevil-Resistance**

Various methods have been attempted to control weevil infestations. These have included leader clipping, shading, application of insecticides and other biological control agents (Tomlin and Borden 1994). Such methods have not been successful or are limited by costs (Tomlin and Borden 1994). Indications that resistance to the white pine weevil

exists in natural populations and may have a genetic basis has led to the hope that it may be possible to utilize this genetic resistance through tree breeding. Exploitation of genetic resistance to insects is widespread in agriculture and forestry (Zas et al. 2005). Breeding for resistance to weevils is not uncommon in agricultural settings. For example, breeding is possible for resistance to the blossom weevil (*Anthonomus rubi*) in strawberry (*Fragaria x ananassa*) (Simpson et al. 2002), to the bean-pod weevil (*Apion godmani*) in common beans (*Phaseolus vulgaris*) (Beebe et al. 1993), and to the bluegrass billbug (*Sphenophorus parvulus*) in range grasses (*Elymus trachyycaulus, E. lanceolatus, Agropyron cristatum, A. desertorum, Psathyrostachys juncea, Leymus salinae*) (Asay et al. 1983). In forestry, breeding for resistance to weevils does not appear to be common, but it is not unheard of. For example, breeding in maritime pine (*Pinus pinaster*) for resistance to the pine weevil (*Hylobius abietis*) is in the beginning stages in Spain (Zas et al. 2005). The breeding program for resistance to the white pine weevil in Sitka spruce is one of the text-book examples for insect resistance breeding in trees (King and Alfaro 2009).

The Sitka spruce breeding program grew out of the resistance first observed on the Green Timbers plantation in Surrey, British Columbia in the 1930s. The striking fact that a few trees showed no damage after a severe attack justified further exploration (King and Alfaro 2009). In the 1970s, the British Columbia Ministry of Forests collaborated with the International Union of Forest Research Organizations (IUFRO) to establish an extensive set of common garden field provenance trials. These trials were set up in 14 locations around British Columbia and included trees from 43 provenances encompassing much of the range of Sitka spruce (Xu et al. 2000). Four different series

were created for different purposes: one for seed source selection, one for preliminary screening for weevil-resistance, and two as components of the IUFRO provenance experiment on Sitka spruce (Ying 1991).

Weevil resistance observed in the IUFRO provenance tests led to the establishment of a small clonal test in 1984 using grafted trees (Ying 1991). The purpose of this clonal trial was to confirm resistant genotypes (Ying 1991). Some interesting patterns were seen in these tests. Trees originating in high weevil hazard zones were less likely to be attacked than those from low hazard zones. Two provenances, Haney (from the lower Fraser Valley) and Big Qualicum (from the east coast of Vancouver Island), were identified as highly resistant pure Sitka spruce sources (Ying 1991).

Armed with this knowledge, new trials were established in 1991-92 to determine whether resistance was more extensively based around these two sources. The idea behind this was that high weevil hazard areas may have selected for a higher proportion of trees with weevil-resistance (King et al. 2004). The outcome of these trials was that the observed weevil-resistance was in fact tightly centered around Haney and Big Qualicum (King et al. 2004). King and Alfaro (2009) report that trees from resistant populations are attacked about half as often as those from other areas. Weevil screening trials that included weevil augmentation were completed, and allowed for the ranking of individual trees according to their weevil resistance (Alfaro et al. 2008).

Individual trees from the screening trials were then chosen as parents for a breeding population (King and Alfaro 2009). From these, 300 full-sib families were created, with the intention of making crosses to test whether different populations have different resistance mechanisms. Since these trials were set up, an effort has been made

to characterize the different populations for their resistance mechanisms. A program led by Dr. John King has ranked individual trees for levels of certain resistance traits, namely constitutive resin canals, traumatic resin canals and sclereids (John King, personal communication).

**Somatic Embryogenesis and Tree Breeding**

Somatic embryogenesis is a technique that allows one zygotic embryo to be multiplied into an indefinite number of somatic embryos through tissue culture. Somatic embryogenesis was first accomplished in conifers in 1985, in Norway spruce (Chalupa 1985 in Park et al. 1993; Hakman et al. 1985) and European larch (*Larix decidua*) (Nagmani and Bonga 1985). Since then, somatic embryogenesis has been achieved in many conifer species (Tautorus et al. 1991). In 1988, Krogstrup first demonstrated the ability of Sitka spruce to undergo somatic embryogenesis.

There are a series of steps involved in creating somatic embryos (Park et al. 1994). First, zygotic embryos are extracted from seeds and placed in contact with a semi-solid nutrient media containing plant growth regulators and organic nitrogen sources. After some time, a percentage of zygotic embryos will show induction of embryogenic tissue. Some cultures will continue to proliferate. When enough tissue has been produced, cultures can be matured into somatic embryos, germinated and grown into somatic seedlings (Park et al. 1994). Somatic embryogenesis is under high genetic control, and it is thought that each stage is controlled by different sets of genes (Park 2002).

The major drive behind somatic embryogenesis technology is the forest industry. Somatic embryogenesis has the potential to be used in clonal forestry, where elite

genotypes could be mass produced. This is being done at scale by companies such as CellFor (Sutton 2002). Somatic embryogenesis has an advantage over traditional cloning methods, such as hedging and grafting, because issues with juvenility can be avoided (Hogberg et al. 1998). Propagation by cuttings can only be done using tree material under a certain age. After that age, the cuttings do not have the ability to root. This means that by the time elite tree genotypes are characterized, they may be too old to be propagated by cuttings. This is not a problem with somatic embryos because tissue can be cryopreserved for any length of time, and thawed to produce embryos once again (Hogberg et al. 1998). This could allow for an indefinite amount of time for screening somatic genotypes. Alternatively, somatic embryogenesis could be used to bulk-up elite genotypes, which could then be used to mass produce cuttings (Park 2002). For these reasons, great effort has been put into improving somatic embryogenesis in conifers, in hopes that it will become a feasible option for propagation (Park 2002).

Studies have been conducted to look at the feasibility of incorporating somatic embryogenesis into breeding programs. One concern is whether the initiation rate will be adequately high enough to include the desired families. Results have been variable. Some studies, such as those by Park et al. (1994) on white spruce, and Hogberg et al. (1998) on Norway spruce, indicate that somatic embryogenesis could work well because initiation rates were good for many families. Other studies, such as that by MacKay et al. (2006) on loblolly pine, suggest that more work is needed to improve induction rates before somatic embryogenesis is feasible. Another concern is whether somatic seedlings will produce trees of the same quality as zygotic seedlings. Studies such as that by Grossnickle and Folk (2007) on interior spruce (*Picea glauca x P. engelmannii*) somatic

seedlings suggest that somatic seedlings meet expectations as plantable seedling crops for variables such as height and diameter growth.

Somatic embryogenesis has previously been incorporated into the Sitka spruce breeding program (Cyr et al. 1999). From 1994 to 1999, material from the Sitka spruce program was used by BC Research Inc. with the intention of developing a clonal program for weevil-resistant lines of Sitka spruce. The goal of this program was to incorporate somatic lines into the selection and use of the resistant material (Cyr et al. 1999). However, this program was terminated and none of the resistant lines were cryopreserved (King and Alfaro 2009).

Somatic embryogenesis has the potential to be useful in breeding programs in other ways. Somatic embryos offer some unique advantages for research, such as the fact that they are grown entirely *in vitro*, therefore in theory eliminating maternal effects (Rohde and Junttila 2008). The environmental conditions during development of the embryo and resulting seedling are tightly controlled. This eliminates much of the environmental variability during development (von Aderkas 2002). This system could be used to ask questions about the stability of phenotypic variation in traits, such as weevil-resistance.

**Introduction to Phenotypic Variation**

The phenotype of an organism is a description of how the organism exists in the world. It is how it looks, functions and behaves. It is the result of the genotype of the organism, that is its DNA, interacting with the environment, at all possible levels, molecular, cellular, and organismal (Sultan 2000). Together, these factors shape the organism. The complexity of these interactions has the potential to be great, but the

flexibility of the organism can also be under certain constraints, making some characteristics more fixed than others (Sultan 2000). These are the interactions that shape the phenotypes that we observe.

Phenotypic variation is intriguing because of its complexity, and a substantial effort has been directed towards dissecting its various components (Sultan 2000; Ackerly et al. 2000; Miner et al. 2005). Phenotypic variation can be considered at different scales. For example, one could look at phenotypic variation over the range of a species. This phenotypic variation considers differences among different genotypes of a species interacting with their environments (Rehfeldt et al. 1999). However, one can also consider possible phenotypic variation within a single genotype, or phenotypic plasticity. This perspective of phenotypic variation considers the flexibility of a single genotype, depending on its interactions with its environment, to arrive at different phenotypes (Miner et al. 2005).

The importance of phenotype is obvious when looked at from an evolutionary perspective. Natural selection acts on phenotypes, favouring those that confer greater fitness, through greater survival and therefore reproductive success (Cody and Mooney 1978). This selection can occur over different time scales, long-term evolution resulting in divergence of species (Charlesworth et al. 1982), and short-term evolution resulting in population differences within species (Silander 1979). Therefore, natural selection helps to shape the patterns of phenotypes over a species' range, a process known as microevolution (Silander 1979). In this way, evolutionary processes are tightly intertwined with phenotypic variation.

**Causes of Phenotypic Variation**

The question of what causes phenotypic variation is complex and is at the root of an extensive body of research stretching from evolution to ecology to development, and beyond (Ackerly et al. 2000; Sultan 2004). Phenotypic variation has implications in many fields from medical genetics (Lander and Schork 1994) to tree breeding (Namkoong 2001). Because of this, understanding the basis of phenotypic variation is a topic of interest in biology.

At the most basic level, phenotypic variance can be divided into the influence of the genotype, the environment, and the interaction between genotype and environment (Schlichting 1986). In fact, the influence of the genotype can be further broken down into those effects that are additive, meaning the effects of a genotype that will be passed on to the offspring, and those effects that are non-additive, such as epistatic effects, the interaction between alleles, or dominance effects, the influence of dominant alleles over recessive alleles. This relationship, between genotype and environment is essential to an understanding of phenotypic variation. The degree to which a given genotype is influenced by the environment in which it develops is variable. Each individual in a population has its own combination of these factors, and this is what results in the phenotype that we observe (Schlichting 1986).

Not all traits are controlled strictly by one or a few genes, and not all traits show discrete phenotypes. Many traits are quantitative, and show a continuum of phenotypes, for example height growth in trees. It is thought that quantitative traits are controlled by multiple genes (Carson and Carson 1989; Lander and Schork 1994). This adds another layer of complexity to phenotypic variation. Traits may be monogenic or polygenic, and may respond to the environment to varying degrees (Carson and Carson 1989).

Phenotypes are what natural selection acts on. If a trait confers some advantage, then that phenotype will be favoured, have greater survival and will reproduce more successfully (Ackerly 2003). In turn, this will increase the frequency of the alleles conferring this phenotype in the population (Ackerly 2003). The trait on which selection acts must be heritable between generations for adaptation to occur. Over very long time scales, natural selection will lead to macroevolution, the evolution of new species (Charlesworth et al. 1982). But, natural selection also works on ecological times scales, between populations within species, leading to microevolution (Cody and Mooney 1978). One of the great examples of this process is the work on Darwin's finches in the Galapagos by Peter and Rosemary Grant (1995). They showed that the shape and size of the finches' beaks changed in relation to short-term changes in food source related to climate fluctuations. Their work shows that the evolution of heritable traits in natural systems can be seen over a relatively short time scale. The products of microevolution are the phenotypes that we observe.

These principles, the interaction of genotype and environment, and evolution by natural selection are considered the basics behind the phenotypes we observe (Schlichting 1986). But these principles do not convey the true complexity behind phenotypic variation. For example, quantitative traits may involve numerous genes; how much each gene contributes to the effect of a trait is variable. For some traits, such as flowering in Arabidopsis, research has shown there are few genes with strong effects (Buckler et al. 2009 and references therein). However, quantitative traits may also be controlled by many genes, each with a relatively small effect (Buckler et al. 2009). This has recently been suggested by an impressive study of maize that involved one million plants. The

authors looked at quantitative trait loci (QTL) in certain adaptive traits, such as time to male and female flowering, and found that they seemed to be controlled by many small-effect QTLs with additive effects. They suggested that this itself is an adaptive mechanism. Since corn is out-breeding, perhaps many small effect genes keep flowering time controlled so that breeding is successfully co-ordinated (Buckler et al. 2009).

This large study of maize also suggested that control of flowering was influenced very little by epistatic effects (Buckler et al. 2009). This means, they found that little of the variation could be attributed to interactions between genes. They reported this as surprising, since epistatic effects have been detected in Arabidopsis and rice (Buckler et al. 2009 and references therein). Epistatic effects are thought to be common in natural populations (Stern and Orgogozo 2009). They can have a great effect on the phenotypic variation seen in a population. Interactions between alleles mean that the same allele bred into one genetic background can produce a completely different effect as the same allele bred into another genetic background. These effects could have opposite directions, one could be adaptive, one could be maladaptive, and this could mean varying fitness, and thus an influence on evolution (Stern and Orgogozo 2009). Further, some genes have effects on multiple traits, known as pleiotropy. Pleiotropic genes in turn can have differing effects in different backgrounds due to epistasis. Therefore the alteration of one gene could have a variety of effects on different traits. This further adds to the complexity behind phenotypic variation (Stern and Orgogozo 2009).

The variability in phenotype that we see across an environment, among populations of a species is also important to consider (Stern and Orgogozo 2009). Greatly differing environments will select for different phenotypes, increasing the

proportion of certain alleles in the population. This is the basis for the adaptation of populations to the environments in which they live. It is also important to consider the history of the populations. Smaller populations may be affected by genetic drift, which is the random fluctuation in alleles. Therefore, a population that was historically small may have reduced variation in a certain trait (Stern and Orgogozo 2009).

It is one thing to look at phenotypic variation over a species or population. But we have to consider another level of phenotypic variation, which is within genotype. Phenotypic plasticity is defined as the ability of a single genotype to produce different phenotypes depending on the environment in which they develop (Pigliucci 1998). Examples of phenotypic plasticity have been reported for many types of organisms, from land plants to insects to small mammals (Sultan 2004), and for many types of traits affecting anatomy, morphology, allocation (Sultan 2000), life history, development and physiology (Sultan 2004). This plasticity in traits is a response to many different types of environmental variables, including abiotic factors such as water or light availability and biotic factors such as predators or competitors (Valladares et al. 2006). The idea of phenotypic plasticity is to look at a single genotype not as a fixed entity, but as a norm of reaction (Sultan 2000).

Phenotypic plasticity has possible important ecological and evolutionary implications. Very high plasticity could allow species to be generalists (Sultan 2004). Plasticity could allow species to live in new environments without having to undergo evolutionary changes by natural selection. Phenotypic plasticity could allow some species to have very large geographic ranges. How "useful" plasticity is depends on how flexible an organism is. For example, very plastic genotypes may allow organisms to live

in very different environments, whereas less plastic genotypes may only allow adjustment to different microhabitats (Sultan 2004).

Taking these factors into consideration, it is clear that phenotypic variation is the result of complex interactions which are difficult to dissect. A wonderful example of this was recently published on the Shrinking Sheep of St. Kilda (Ozgul et al. 2009). Here, the authors used a technique known as Price's equation to pull apart all the various contributing factors to a trend in a heritable trait, the weight of the sheep. It appeared over many years, that the sheep were getting smaller, despite selection for larger sheep. The sheep were being selected for larger size, but this factor was counteracted by other factors. In particular, the birth weights of offspring were on average smaller than the birth weights of their mothers. Furthermore, the climate had fluctuated over the course of the experiment, and as a result more slow-growing sheep were surviving the winters. Ozgul et al. (2009) showed that the phenotypes that we see cannot always be explained in simple terms.

**Phenotypic Variation in Conifers**

A number of conifer species have very wide distributions, encompassing a range of geographical and climatic factors. For example, white spruce has an enormous range, stretching across much of northern North America (Li et al. 1997), and Scots pine has a range that stretches across Europe and Asia (Shutyaev and Giertych 2000). As a result of these wide ranges, over time conifer species have encountered many selective forces, both abiotic and biotic, that have caused conifer species to show adaptations to their local environments (de Groot and Schnekenburger 1999).

Phenotypic variation in conifers has been recognized for centuries for the simple reason that wood is a valuable resource. It was known in the 18th century that different sources of seed could produce trees with different wood characteristics (Matyas 1996). For example, Duhamel du Monceau, the inspector-general of the French navy and botanist realized this, and set up an experiment where he planted Scots pine from different provenances in the Baltics, for the purpose of finding sources of seed that would produce trees suitable for ship-building. In the 1820s, Andre de Vilmorin set up similar studies with Scots pine. In 1862, Vilmorin published the results of these trials and suggested that different provenances showed differences in their characteristics. Vilmorin's results were found to be so interesting by his forestry contemporaries that he inspired many other trials to be established throughout Europe. In fact, when the Union of Forest Research Institutions (IUFRO) was established in 1892, one of its first efforts was to set up international provenance experiments. Since then, many programs have been established, investigating many traits, and it is the results of these programs that have provided the foundation for research into phenotypic variation in conifers (Matyas 1996).

In general, trials are set up to understand how trees originating in different places develop when placed in the same environment (Matyas 1996). Such experiments are known as common garden experiments. Trials are repeated in different environments, which allow the responses of different sources to be seen, and the genetic and environmental components of the phenotypic variation to be pulled apart. The main purpose of these trials is to better understand traits relevant to forestry (Matyas 1996). Traits such as height growth and vigor (Samuel 1991), as well as wood quality properties

(St. Clair 1994) are often the focus. Some trials are aimed at screening many provenances for these traits (Danjon 1994), while others have much more narrow goals, such as screening trees for specific wood qualities (Hansen and Roulund 1998). Once desirable sources are found, tree breeders must determine whether such traits are heritable. If the desired traits are heritable, then tree breeders are able to selectively breed trees and produce seeds that are considered improved. Even though the ultimate goal of tree breeding experiments is to domesticate forest trees and allow for ever faster and better growing trees (de Groot and Schnekenburger 1999), the scope of many of these experiments is huge and thus they also reveal information about the patterns underlying phenotypic variation in conifer species (Ying and Yanchuk 2006).

Numerous experiments have shown patterns of variation related to climate, through correlations with altitude and latitude. For example, in Ponderosa pine, growth potential decreases as elevation increases. This was linked to the number of frost-free days at different elevations (Rehfeldt 1991). In white spruce, northern populations flush earlier and set bud earlier, whereas southern populations show superior growth (Lesser and Parker 2004). In other species, genetic variation related to clines has been seen in frost hardiness (Johnsen and Skroppa 2000), seed maturation (Harju et al. 1996), wood density (Lesser an Parker 2004), and many other traits. It is also known that some conifer species have adapted numerous times to climate changes caused by glaciations (Cwynar and MacDonald 1987), and these changes have also left their mark on conifer populations and in the phenotypic variation (and underlying genotypic variation) that is seen. However, it is recognized that it is not just climate or geography alone that explains the

phenotypic variation. It is also the complex interactions between these factors and between local peculiarities that results in phenotypic variation (Rehfeldt 1991).

Some of the best examples of local peculiarities come from examples of breeding trees for resistance to pests and pathogens. Some trials have shown provenance differences in resistance to certain pests. For example, Yanchuk et al. (2008) have shown possible provenance differences in resistance to the mountain pine beetle in lodgepole pine. Other trials show resistance that has a much patchier, discontinuous pattern of variation. For example, in Sitka spruce, the resistance found to the white pine weevil is tightly centered around two sources, representing a fraction of its range (King et al. 2004). Other similar examples exist, such as resistance to fusiform rust in some populations of loblolly pine in Mississippi, which also had a very centralized resistant population (Wells et al. 1982). Such examples of pest and pathogen resistance demonstrate that local adaptations to local selective forces are also at work in shaping patterns of phenotypic variation.

The overarching idea that can be taken from these examples is that the phenotypic variation that we see in conifer species is partly the result of their evolutionary response to the environment in which they have evolved, including all of the historical events acting on the different populations, by means of genetic drift or bottlenecks. This becomes more complex if we consider that many of the traits studied in these trials are quantitative, and likely controlled by many genes (Wheeler et al. 2005). This leads to the possibility that such traits are linked genetically. For example, genetic correlations between budset and frost hardiness have been identified (Johnsen and Skroppa 2000).

Johnsen and Skroppa (2000) suggest that pleiotropic effects may be at work in quantitative traits in forest trees.

It is tempting to want to understand the evolutionary mechanisms behind phenotypic variation in conifers, but this is a difficult task. Conifers are long-lived organisms, making it difficult or impossible to study survival. They also take many years to reach reproductive maturity, which limits estimates of fitness. Even estimates of trait heritability are hindered. The costs alone of setting up and maintaining long-term trials of conifers is prohibitive. This means that studies of evolution in a traditional sense, that is observing multiple generations such as the Grant's work on finches, is not possible with conifers. Any understanding of evolution in conifers must be inferred.

However, there has been work on the genetic mechanisms underlying phenotypic variation through molecular studies. Here, the focus is ultimately on identifying genes responsible for traits of interest. A number of different approaches have been taken, each with their own advantages and disadvantages. Searching for quantitative trait loci (QTLs) is a technique where phenotype is used to locate regions of the genome that affect the trait (Grattapaglia et al. 2009). QTLs are useful for looking at the genes underlying complex traits when it is difficult to find candidate genes, such as in conifers (Grattapaglia et al. 2009). QTL studies in conifers have included the use of RFLPs, RAPDs, AFLPs, and SSRs (Namroud et al. 2008; Grattapaglia et al. 2009). A number of studies have used QTL analysis to understand traits of forest trees, and the results suggest that the traits are controlled by a few QTLs of modest effect (Wheeler et al. 2005). It was hoped that QTLs would help decipher the genetics underlying complex adaptive and economic traits (Wheeler et al. 2005). Neale and Ingvarsson (2008), however, suggest

that using QTLs to understand complex adaptive traits in forest trees is difficult because the resolution of QTLs is too low to allow the genes underlying the traits to be detected. The genes underlying phenotypic variation may be more easily deciphered using other approaches such as association genetics. Neale and Ingvarsson (2008) suggest that forest trees might even be an unusual model system because conifers exist in both undomesticated and partly domesticated systems, meaning the extensive network of provenance studies, and that this may aid in the identification of genes. Other types of molecular genetics approaches are also underway, including genome scans (Namroud et al. 2008) and comparative and population genomics. These studies may help to elucidate the genetic basis of phenotypes in conifers, but the consensus seems to be that genetic studies of conifers will continue to be challenging owing to their very large genome sizes and the resulting lack of genetic databases for conifers (Namroud et al. 2008).

There is another level at which to look at phenotypic variation in conifers, and that is at the level of the individual genotype. Phenotypic plasticity considers the effect of environment on individual genotypes, and the resulting phenotypic variation. To study phenotypic plasticity at this level requires a clonal system. This reduces the effect of additive and heritable variance, allowing the resulting effect to be attributed mainly to environmental variance (von Aderkas 2002). Somatic embryos can provide a useful clonal system in conifers. Von Aderkas (2002) used larch somatic embryos to show that there is phenotypic plasticity in cotyledon number *in vitro*, and that this variation can be controlled by the environment in which the embryos develop, in this case by different levels of plant growth regulators. In another example, von Aderkas et al. (2007) demonstrated that interior spruce somatic embryo clonal lines showed phenotypic

plasticity in cold tolerance, again by altering external factors during development, in this case with temperature. These examples further the point that the phenotypic variation that we observe in conifers, whether they are embryos or trees, is a result of complex interactions between the genotype and the environment.

**Project Aim**

Results from the Sitka spruce breeding program have shown that there is phenotypic variation in levels of resistance to the white pine weevil and that this resistance is heritable (King et al. 2004). It has also been suggested that the mechanisms of resistance vary between different resistant genotypes (King and Alfaro 2009). Through screening and anatomical study, genotypes with varying levels of these traits, namely resin canals and sclereid cells, have been identified. What is not known, is how consistent these traits are within genotype. By using controlled pollinations between selected genotypes ranked for these resistance traits we can capitalize on genotypic variation in these traits, and then by creating clonal lines of the resulting progeny through somatic embryogenesis, we can study the consistency of these traits in clones. The goal of this study is to use somatic embryogenesis to set up a system that can be used to explore the phenotypic and genotypic variation in resistance traits to the white pine weevil in Sitka spruce.

# Chapter 2: Creating a Somatic Embryogenic System

## Introduction

Phenotypic variation exists between genotypes of a species, but phenotypic variability may exist within genotypes as well (Valladares et al. 2006; Sultan 2004). In tree breeding, the goal is to understand and capitalize on traits that occur in nature. Tree breeders work to understand the phenotypic variation between individuals regarding useful traits, and to take advantage of it (Lee et al. 2002). This is the case with Sitka spruce (Ying 1991; King et al. 2004). Through the Sitka spruce breeding program, resistance traits to the white pine weevil have been identified in individuals from certain populations (King and Alfaro 2009). These traits include sclereid cells, constitutive resin canals and traumatic resin canals. Genotypes have been ranked for the abundance of these traits, and individuals of different rankings have been identified, replicated through grafting, and established in breeding orchards. Now that trees are of seed-bearing age and have been extensively characterized (King and Alfaro 2009), this breeding program may provide a valuable resource for understanding the phenotypic variation present for these resistance traits.

While phenotypic variation between genotypes may be studied relatively easily, studying phenotypic variation within genotypes is a challenge because it requires clones. Sitka spruce can be cloned a number a ways, including grafting (Barnett and Miller 1994), rooted cuttings (van den Driessche 1997) and by somatic embryos. Somatic embryogenesis is advantageous because it allows development to be followed from embryo to mature tree (Filonova et al. 2000).

Genetically identical embryos and trees derived through somatic embryogenesis can be used to study phenotypic variability within genotypes. Clonal replicates make drawing comparisons of traits between genotypes more robust (Burr and Tinus 1996). My first goal was to develop a system that can be used to study phenotypic variability in resistance traits to the white pine weevil in Sitka spruce. My approach was to create SE clonal lines using material from the British Columbia Sitka spruce breeding program. I wanted to produce multiple SE lines using the progeny of controlled crosses from parent trees having extreme phenotypes for specific resistance traits.

Cryopreservation

An advantage of using an SE system is that tissue can be cryopreserved in liquid nitrogen indefinitely. Tissue can be recovered from cryostorage and re-established into an embryogenic culture. This is seen as an advantage by tree breeders because genotypes can be cold-stored while field testing of potential elite genotypes takes place (Park et al. 1994, 1998; Park 2002). The advantage extends to research, as clones can be reproduced for experiments as needed. This is different from traditional propagation techniques, like grafting, which are constrained by the need to maintain juvenility in the parent plants. Often parent plants pass the juvenile stage before field testing can occur (Park et al. 1998). Simple methods of cryopreservation are crucial if SE technology is to be integrated into tree breeding.

Here, I tested a novel method of cryopreservation. This method is simple, only requiring pretreatment on maturation medium containing ABA and PEG, and a temperature treatment at 5 °C. This is different from traditional methods that require chemical pretreatment steps with sorbitol and DMSO, and stepwise cooling in a

programmable freezer (Percy et al. 2001; Kartha et al. 1988; Klimaszewska et al. 1992; Cyr et al. 1994). The SE material from our Sitka spruce project provided an opportunity to test this method.

## Methods and Materials

### Breeding

Plant Material

Sitka spruce (*Picea sitchensis*) cones were provided by Dr. John N. King through the British Columbia Ministry of Forests and Range Sitka spruce Weevil-Resistance Breeding Program. The cones are the result of an extensive screening and breeding program for resistance to the white pine weevil (*Pissodes strobi*) (King and Alfaro, 2009).

Controlled pollinations were conducted in May 2008 by Dr. John N. King and David Ponsford. The controlled pollinations took place at four locations: BC Ministry of Forests Puckle Road Research Station (Saanichton, BC, Canada), Yellow Point Propagation (Ladysmith, BC, Canada), Western Forest Products Saanichton Forestry Centre (Saanichton, BC, Canada), BC Ministry of Forests Cowichan Lake Research Station (Mesachie Lake, BC, Canada). We used parent genotypes previously characterized for the abundance of three physical resistance traits: sclereids, traumatic resin canals and constitutive resin canals (for methods, see Grau 2006). Genotypes with the most extreme phenotypes for these traits were selected for crossing, based on parental clonebank data (provided by Dr. John King, personal communication). Crosses were made where both the male and female parents ranked high for a trait, and where both male and female ranked low for a trait. For the sclereid and traumatic resin canal traits, we also made crosses where one parent ranked high for a trait, and one parent ranked low for a trait. Also, we chose genotypes originating from the Queen Charlotte Islands as controls.

We began collecting cones two months after pollination. Up to six cones were collected per cross from a total of 30 crosses and from 4 open-pollinated genotypes. Cones were transported on ice and stored for up to three wks at 5 ± 1 °C.

**Induction**

Scales were pulled individually from each cone and seeds were removed from the scales with forceps. Seeds were surface-sterilized in 20 % Javex solution (Javex-12, 12.6 % w/v sodium hypochlorite, Colgate-Palmolive, Toronto, Canada) for 10 min and rinsed three times with sterile deionized water. Steps were carried out in a laminar airflow hood using sterilized equipment. Seeds were emptied onto sterile Petri plates for dissection.

With the aid of a Leica WILD M3C dissecting microscope, either the entire megagametophyte or the embryo was removed depending on the developmental stage of the seed. If the embryo was large enough to be seen, it was removed. Explants were placed onto induction medium in Petri dishes (100x15 mm, Fisherbrand, Fisher Scientific, USA).

The basal induction medium consisted of half-strength modified Litvay's medium (Litvay et al. 1985) supplemented with sucrose (10 g/L) and casein hydrolysate (0.8 g/L). The medium was adjusted to pH 5.8 prior to autoclaving. Glutamine (0.4 g/L), 2,4-dichlorophenoxyacetic acid (20 µM) and benzylaminopurine (10 µM) were filter-sterilized and added to the medium following autoclaving. The medium was solidified with 3.2 g/L Phytagel (Sigma, St. Louis, MO, USA). Petri plates were sealed with plastic film (No Name 90m, Loblaws, Toronto, Canada) and kept in darkness at 22 ± 1 °C. Dissections were carried out over six weeks in July and August 2008.

Nine weeks from the first dissections, plates were screened using a dissecting microscope for the appearance of embryogenic tissue. Screening continued bi-weekly for two months. Successfully induced, i.e. embryogenic material, was placed on maintenance medium. Maintenance medium was similar to induction medium with the exception that the hormone supplements were 10 μM 2,4-dichlorophenoxyacetic acid and 5 μM benzylaminopurine only. Plates were sealed with plastic film and kept in darkness at 22 ± 1 °C.

Explants with embryogenic tissue that continued to proliferate once placed on maintenance medium were monitored. These lines were subcultured onto fresh maintenance medium bi-weekly to increase the amount of tissue. Necrotic tissue was removed at each subculture.

**Maturation**

Lines producing adequate amounts of embryogenic tissue by 12 weeks following first transfer to maintenance medium were selected for maturation treatment. Maturation medium consisted of half-strength modified Litvay's medium supplemented with sucrose (30 g/L ) and casein hydrolysate (0.8 g/L). The pH of the medium was adjusted to 5.8 before autoclaving. Abscisic acid (50 μM) and glutamine (0.4 g/L) were filter-sterilized and added to the medium after autoclaving. The medium was solidified with 5 g/L Phytagel.

Pieces of embryogenic tissue (1 cm diameter) were placed on to maturation medium. The tissue was gently spread into a thin layer using forceps. Plates were sealed with plastic film and stored in darkness at 22 ± 1°C. Depending on the availability of tissue, up to six plates were made for each line.

After eight weeks, screening for mature embryos took place. Embryos were considered mature when they had developed cotyledons. When the majority of embryos of a particular genotype had cotyledons, the culture was moved to a darkened growth chamber at 5 °C to arrest development temporarily. Screening continued bi-weekly for a further two months.

The ability of each line to mature was estimated by classifying the culture according to the approximate number of mature embryos per plate as 0, 1-10, 10-30 or >30. These approximations were necessary to avoid damaging or contaminating embryos by opening the plates.

**Germination**

Genotypes were selected for germination using two criteria. First, I selected genotypes to evenly represent the crosses for the three resistance traits; I also selected from control crosses. Within this framework, I selected genotypes that produced adequate numbers of good quality embryos. Good quality embryos were defined as opaque embryos with at least three cotyledons.

The medium used for germination was half-strength modified Litvay's medium supplemented with ammonium phosphate (1 g/L), sucrose (10 g/L), casein hydrolysate (0.8 g/L) and fine activated charcoal (5 g/L). The medium was adjusted to pH 5.8 before autoclaving. Glutamine (0.4 g/L) was filter-sterilized and added to the medium after autoclaving. The germination medium was solidified with 3.2 g/L of Phytagel in Petri plates.

The best quality embryos from each plate were individually selected and placed so that the long axis of the embryo touched the surface of the germination medium. For

each genotype, up to three plates of 16 embryos were made. Plates were sealed with plastic film. The plates were placed in darkness at 22 ± 1 °C for one wk. Plates were then arranged in a single layer and exposed to light (35 μE $m^{-2}$ $s^{-1}$, 16 h photoperiod) for two months.

**Acclimatization**

Once germinants had developed primary leaves, they were planted in miniplug blocks (Series 400, Beaver Plastics, Acheson, Alberta, Canada) containing cellular rooting sponge medium (Product Number 400, Cellular Rooting Sponge Technology, Grow-Tech, Lisbon Falls, ME, USA). Prior to planting, the miniplug blocks were submerged in water for 24 h to wet the medium. I pasteurized the miniplug blocks by saturating them with boiling water and sealing them in a plastic bag for 15 minutes. This procedure was repeated once. Blocks were cooled to room temperature. Germinants were placed into the plug cavities using smooth-tipped forceps. They were positioned so the cotyledons were just above the surface of the medium. The planted germinants were frequently sprayed with water to prevent desiccation. Each block was watered thoroughly after planting.

The blocks were placed into a fogging chamber in the glasshouse of the Bev Glover Greenhouse Research Facility at the University of Victoria. The frame of the fogging chamber was constructed with PVC pipe. It measured 160 cm length by 82 cm depth and 87 cm peak height. The frame was covered with 6 mil polyethylene greenhouse film, then sealed with polyethylene patch tape. Each peak had a 100 $cm^2$ vent. Inside the chamber, two ultrasonic water foggers (Mist Maker, Model DL24,

Foshan Nanhai Techsin Electric Co., Xiabei, China) were placed in trays of water to humidify the air.

Until roots had grown, the relative humidity was kept at 90 ± 5 %. The temperature was kept below 30 °C. The germinants were gradually exposed to light by removing layers of shade cloth. Once roots were visible, the relative humidity was decreased to 65 ± 5 %. The germinants were watered as needed and fertilized bi-weekly with 50 ppm of 20:8:20 N:P:K (High Nitrate Forestry Seedling Special, Plant-Prod, Brampton, ON, Canada).

In response to a fungus gnat (Sciaridae) infection, the blocks were treated with insect parasitic nematodes (*Steinernema feltiae*) (Koppert Biological Systems, Netherlands). These nematodes attack the larvae of the fungus gnats. Sticky-card traps (Chromaline Card Traps, Phero Tech Products, Delta, BC) were used to control adult flies.

**Growth**

When the plants reached a height of 4 cm and the shoots had become dark green, they were replanted into styroblock containers (Superblock 412A, Beaver Plastics, Acheson, Alberta, Canada). The styroblocks were sterilized using 4.5 % Javex-12 solution for 5 min and rinsed thoroughly with tap water. We used moistened Sunshine Basic Professional Growing Mix (Sun Grow Horticulture, Vancouver, BC, Canada) and peat moss in a ratio of 9:1 as our growing medium. After transplanting, the growing medium was saturated with water. The top of the medium was covered with a layer of granite aggregate to prevent growth of algae and mosses.

Styroblocks were placed in a glasshouse room equipped with a misting system. The relative humidity was kept at 70 ± 5 %. The temperature was kept above 20 °C. The plants were watered as needed. They were fertilized bi-weekly with 50 ppm of 20:8:20 N:P:K (High Nitrate Forestry Seedling Special, Plant-Prod, Brampton, ON, Canada). When roots reached the bottom of the block cavities, the relative humidity was reduced to 50 ± 5 %. The temperature, water and fertilizer treatments remained the same. At the end of October 2009, artificial light was supplied for 15 hours per day.

**Cryopreservation**

Pretreatment

Approximately 2 g of embryogenic tissue was added to 10 mL of sterile liquid medium in a sterile centrifuge tube. The liquid medium consisted of half-strength modified Litvay's medium supplemented with sucrose (30 g/L) and casein hydrolysate (0.8 g/L). The pH of the medium was adjusted to 5.8 before autoclaving. The tissue and medium were shaken to evenly suspend the tissue.

This suspension was spread on to Petri plates filled with modified maturation medium. The modified maturation medium consisted of half-strength modified Litvay's medium supplemented with sucrose (30 g/L), casein hydrolysate (0.8 g/L) and polyethylene glycol 4000 (50 g/L). The pH of the medium was adjusted to 5.8 before autoclaving. Abscisic acid (50 µM) and glutamine (0.4 g/L) were filter-sterilized and added to the medium after autoclaving. The medium was solidified with 5 g/L Phytagel (Sigma, St. Louis, MO, USA).

Two plates were made for each genotype. Plates were sealed with plastic film and stored at 22 ± 1 °C for two days. The plates were then moved to a darkened growth

chamber at 5 °C. After 10 wks, the response of each genotype to the pretreatment was ranked, based on growth and appearance of maturing embryos.

Cryopreservation

After four months, the cultures were processed for cryopreservation. Tissue was scraped from the plates with a sterile scupula and placed into sterile cryovials (2 mL Nunc CryoTube Vials, Nunc Brand Products, Nalge Nunc International, Denmark). Two vials were prepared for each genotype. The vials were submerged directly in liquid nitrogen.

Since this method was new, it required verification. All cryopreserved genotypes were tested for survival and continued embryogenicity. I removed one vial per genotype after 24 hrs in liquid nitrogen. The vials were immersed in a water bath at 39 ± 1 °C for 3 min. Tissue was gently spread with forceps on maintenance medium. Plates were sealed with plastic film and stored in darkness at 22 ± 1 °C. Plates were screened for tissue growth after four weeks. Tissue growth was recorded according to one of the following categories: embryogenic, non-embryogenic callus, or dead.

**Histology**

Plant Material - Developmental Series

I collected samples of somatic embryos throughout development. I used an embryogenic line of Sitka spruce (OL6) which was known to produce high quality embryos. This embryogenic line was induced as part of this project. Samples were taken weekly for seven wks from cultures placed on maturation medium.

Fixing, Embedding, Sectioning

All samples were fixed with 2.5 % glutaraldehyde in 0.05 M phosphate (K/K) buffer (pH 7.2). The samples were stored at 5 ± 1 °C. Samples were each rinsed five times in 0.05 M phosphate buffer (pH 7.2) for 30 min. I dehydrated the samples in an ethanol series (30 %, 50 %, 70 %, 90 %, 95 %). Each step was 30 min in duration. The last dehydration step was repeated once. Samples were pre-infiltrated for 24 h with a solution of equal amounts of 95 % ethanol and Technovit 7100 base liquid (Heraeus Kulzer GmbH, Wehrheim, Germany). Samples were then infiltrated with Technovit 7100 preparation solution consisting of 1 % w/v Hardener I in Technovit 7100 base liquid. Samples were infiltrated for at least ten days. During this time, the preparation solution was changed every other day.

Samples were embedded in Technovit 7100 base liquid and Hardener II in a ratio of 15:1. Embedding trays and resin solution were chilled on ice. Sheets of acetate were used to exclude air from the resin during curing. Samples were cured at room temperature. We mounted all samples using Technovit 3040 prepared by mixing 2 g of the powder component with 1 mL of the liquid resin component. Samples were sectioned using a Leica SM2400 sledge microtome with a steel tungsten carbide blade.

Longitudinal sections (5 μm thick) were made for each developmental stage. Sections were placed onto droplets of water and baked on to slides at 60 °C until all water evaporated.

Toluidine Blue Staining

Sections were bleached (10 s) and rinsed three times (30 s each) in water. A blast of canned air was used to dry the sections. They were then stained for 4 min with 0.05 %

(w/v) Tol blue (in distilled water) and rinsed with water until the plastic around the specimen was destained. Sections were dried with a blast of canned air (Gutmann 1995).

**Statistical Methods**

Proportions for induction and germination rates were arcsine transformed using the function: arc p = ASIN (SQRT(p)), where p is the proportion. Values of 0 were corrected before arcsine transformation using the formula: p = 1 / (4 * n), where n is the number of samples (Snedecor and Cochran 1980). Mean values were back-transformed.

## Results

### Breeding and Induction

The mean number of filled seeds per cone was 9.4 ± 6.8 (mean ± SD) for the control-pollinated crosses (Table 1). The controlled-pollinations were not as successful as expected. Every filled seed available was used as an explant for induction. Therefore, the number of filled seeds per cross also represents the number of zygotic embryos used as explants for induction. There were many more filled seeds available in the open-pollinated crosses. I only extracted 40 seeds per cone from these crosses (Table 2), but there were also additional filled seeds. In total, I plated 1520 explants onto induction medium, representing 30 control-pollinated crosses and 440 explants from four open-pollinated genotypes (Table 1, 2).

The overall mean induction rate was 7 % (Table 3). Induction rates per cross were spread over a wide range, from 0 % to 56 %. When grouped into the resistance trait categories, differences in mean induction rates were evident. The constitutive resin canal crosses had the highest mean induction rate at 15 %. The induction rates for these crosses ranged from 1 % to 56 %. The sclereid cell crosses were the second most successful group. The induction rate for this group was 7 %, about half that of the constitutive resin canal crosses. The range in induction rates for sclereid cell crosses was from 0 % to 33 %. Both the traumatic resin canal crosses and the control crosses had induction rates similar to the sclereid cell crosses, at 6 % and 5 % respectively. Their induction rates had narrower ranges. Traumatic resin canal crosses had induction rates ranging from 0 % to 8 %. The induction rates for the control crosses ranged from 1 % to

**Table 1. Number of filled seeds per cone for controlled-pollinations of Sitka spruce.** Cones were collected between June and August 2008. Parent genotypes were previously ranked for abundance of sclereids, traumatic resin canals, or constitutive resin canals. High and low refer to the ranking for the given parent and the given trait. Cones were also collected from control crosses with parent genotypes originating on the Queen Charlotte Islands.

**Table 1**

| Sclereid cells | Cross* | Code** | Number of Cones | Number of Filled Seeds | Mean Number of Filled Seeds per Cone |
|---|---|---|---|---|---|
| **High female, high male** | 1020x1153 | BC | 2 | 27 | **14** |
| | 946x1020 | AB | 6 | 48 | **8** |
| | 1020x1242 | BD | 6 | 43 | **7** |
| | 1242x1020 | DB | 6 | 24 | **4** |
| | 1020x946 | BA | 6 | 22 | **4** |
| | 1242x946 | DA | 6 | 8 | **1** |
| **High female, low male** | 1242x1018 | DF | 6 | 73 | **12** |
| **Low female, high male** | 1018x1020 | FB | 6 | 37 | **6** |
| **Low female, low male** | 860x1018 | EF | 6 | 87 | **15** |
| | 1018x1253 | FG | 6 | 39 | **7** |
| | 1253x1018 | GF | 6 | 20 | **3** |
| | 1018x1018 | FF | 2 | 4 | **2** |
| | 1253x860 | GE | 6 | 10 | **2** |
| **Traumatic Resin Canals** | | | | | |
| **High female, high male** | 921x889 | JH | 6 | 41 | **7** |
| | 921x1010 | JI | 2 | 4 | **2** |
| | 889x1010 | HI | 6 | 1 | **0** |
| **High female, low male** | 921x1075 | JK | 6 | 107 | **18** |
| **Low female, high male** | 1075x889 | KH | 6 | 12 | **2** |
| | 1075x1010 | KI | 6 | 23 | **4** |
| **Low female, low male** | 1075x945 | KL | 6 | 41 | **7** |

**Table 1 continued . . .**

| Constitutive Resin Canals | Cross* | Code** | Number of Cones | Number of Filled Seeds | Mean Number of Filled Seeds per Cone |
|---|---|---|---|---|---|
| **High female, high male** | 1043x1018 | MF | 6 | 123 | **21** |
| | 1209x1018 | NF | 4 | 64 | **16** |
| | 1043x1209 | MN | 6 | 78 | **13** |
| **Low female, low male** | 1241x1010 | OI | 4 | 82 | **21** |
| | 1241x945 | OL | 4 | 44 | **11** |
| | 1010x945 | IL | 6 | 65 | **11** |
| **Controls** | | | | | |
| | 276x444 | QR | 6 | 151 | **25** |
| | 437x444 | SR | 6 | 124 | **21** |
| | 215x444 | TR | 6 | 78 | **13** |
| | 215x437 | TS | 6 | 40 | **7** |
| | | | **Mean number of filled-seeds per cone for control-pollinations** | | **9.4** |
| | | | | **Standard Deviation** | **6.8** |

* Refers to parent genotype numbers as assigned by the BC Ministry of Forests Weevil Resistance Breeding Program. The first number refers to the female parent genotype. The second number refers to the male parent genotype.

** Letters also refer to the parent genotypes of the crosses. The first letter refers to the female parent genotype. The second letter refers to the male parent genotype. These codes were assigned for ease of labelling. They are used to refer to genotypes throughout this project.

**Table 2. Number of seeds collected per cone for open-pollinated Sitka spruce.** Cones were collected between June and August 2008.

**Table 2**

| Open-pollinated | Maternal Parent | Code** | Number of Cones | Number of Filled Seeds Extracted per Cone*** |
|---|---|---|---|---|
| | 1020 | B | 4 | **40** |
| | 1018 | F | 2 | **40** |
| | 1010 | I | 3 | **40** |
| | 1043 | M | 2 | **40** |

* Refers to the female parent genotype number as assigned by the BC Ministry of Forests Weevil Resistance Breeding Program.

** Letters also refer to the parent genotypes of the crosses. The letter refers to the female parent genotype. These codes were assigned for ease of labelling. They are used to refer to genotypes throughout this project.

*** Unlike the control-pollinated crosses, the number of filled seeds was not limiting in the open-pollinated crosses. I stopped extracting seeds after reaching 40 seeds per cone.

**Table 3. Induction rates for somatic embryogenic cultures from Sitka spruce.** Cultures were derived from control-pollinated crosses with parent genotypes previously ranked for abundance of sclereids, traumatic resin canals, or constitutive resin canals. High and low refer to the ranking for the given parent and the given trait. Cultures were also derived from control crosses with parent genotypes originating on the Queen Charlotte Islands, and from open-pollinated crosses.

**Table 3**

| Sclereid Cells | Cross | Code | Number of Uncontaminated Explants | Established Cultures | Arcsine Transformed Proportions |
|---|---|---|---|---|---|
| **High female, high male** | 1020x1242 | BD | 43 | 0 | **0.08** |
| | 1020x946 | BA | 20 | 0 | **0.11** |
| | 1020x1153 | BC | 27 | 1 | **0.19** |
| | 946x1020 | AB | 48 | 5 | **0.33** |
| | 1242x946 | DA | 8 | 1 | **0.36** |
| | 1242x1020 | DB | 24 | 8 | **0.62** |
| **High female, low male** | 1242x1018 | DF | 73 | 4 | **0.24** |
| **Low female, high male** | 1018x1020 | FB | 35 | 2 | **0.24** |
| **Low female, low male** | 1018x1018 | FF | 4 | 0 | **0.25** |
| | 1253x860 | GE | 9 | 0 | **0.17** |
| | 1018x1253 | FG | 38 | 4 | **0.33** |
| | 1253x1018 | GF | 19 | 2 | **0.33** |
| | 860x1018 | EF | 74 | 9 | **0.36** |
| | | | | **Mean** | **0.28** |
| | | | **Back transformed mean proportion** | | **0.07** |
| | | | | **Range** | **0.00-0.33** |

**Table 3 continued . . .**

| Traumatic Resin Canals | Cross | Code | Number of Uncontaminated Explants | Established Cultures | Arcsine Transformed Proportions |
|---|---|---|---|---|---|
| **High female, high male** | 889x1010 | HI | 1 | 0 | **0.52** |
| | 921x889 | JH | 41 | 0 | **0.08** |
| | 921x1010 | JI | 4 | 0 | **0.25** |
| **High female, low male** | 921x1075 | JK | 97 | 1 | **0.10** |
| **Low female, high male** | 1075x889 | KH | 12 | 0 | **0.14** |
| | 1075x1010 | KI | 23 | 1 | **0.21** |
| **Low female, low male** | 1075x945 | KL | 40 | 7 | **0.43** |
| | | | | **Mean** | **0.25** |
| | | | | **Back transformed mean proportion** | **0.06** |
| | | | | **Range** | **0.00-0.08** |

**Table 3 continued . . .**

| Constitutive Resin Canals | Cross | Code | Number of Uncontaminated Explants | Established Cultures | Arcsine Transformed Proportions |
|---|---|---|---|---|---|
| **High female, high male** | 1043x1018 | MF | 108 | 1 | **0.10** |
| | 1209x1018 | NF | 63 | 7 | **0.34** |
| | 1043x1209 | MN | 77 | 11 | **0.39** |
| **Low female, low male** | 1241x1010 | OI | 75 | 7 | **0.31** |
| | 1241x945 | OL | 44 | 7 | **0.41** |
| | 1010x945 | IL | 59 | 33 | **0.84** |
| | | | | **Mean** | **0.40** |
| | | | | **Back transformed mean proportion** | **0.15** |
| | | | | **Range** | **0.01-0.56** |
| **Controls** | | | | | |
| | 276x444 | QR | 151 | 2 | **0.12** |
| | 437x444 | SR | 119 | 6 | **0.23** |
| | 215x437 | TS | 40 | 2 | **0.23** |
| | 215x444 | TR | 78 | 9 | **0.35** |
| | | | | **Mean** | **0.23** |
| | | | | **Back transformed mean proportion** | **0.05** |
| | | | | **Range** | **0.01-0.12** |

**Table 3 continued . . .**

| Open-pollinated | Maternal Parent | Code | Number of Uncontaminated Explants | Established Cultures | Arcsine Transformed Proportions |
|---|---|---|---|---|---|
| | 1018 | F | 80 | 1 | **0.11** |
| | 1043 | M | 65 | 1 | **0.12** |
| | 1020 | B | 158 | 3 | **0.14** |
| | 1010 | I | 113 | 7 | **0.25** |
| | | | | **Mean** | **0.16** |
| | | | | **Back transformed mean proportion** | **0.02** |
| | | | | **Range** | **0.01-0.06** |
| | | | | **Mean of arcsine values (all values)** | **0.27** |
| | | | | **Back transformed mean proportion (all values)** | **0.07** |
| | | | | **Range (all values)** | **0.00-0.56** |

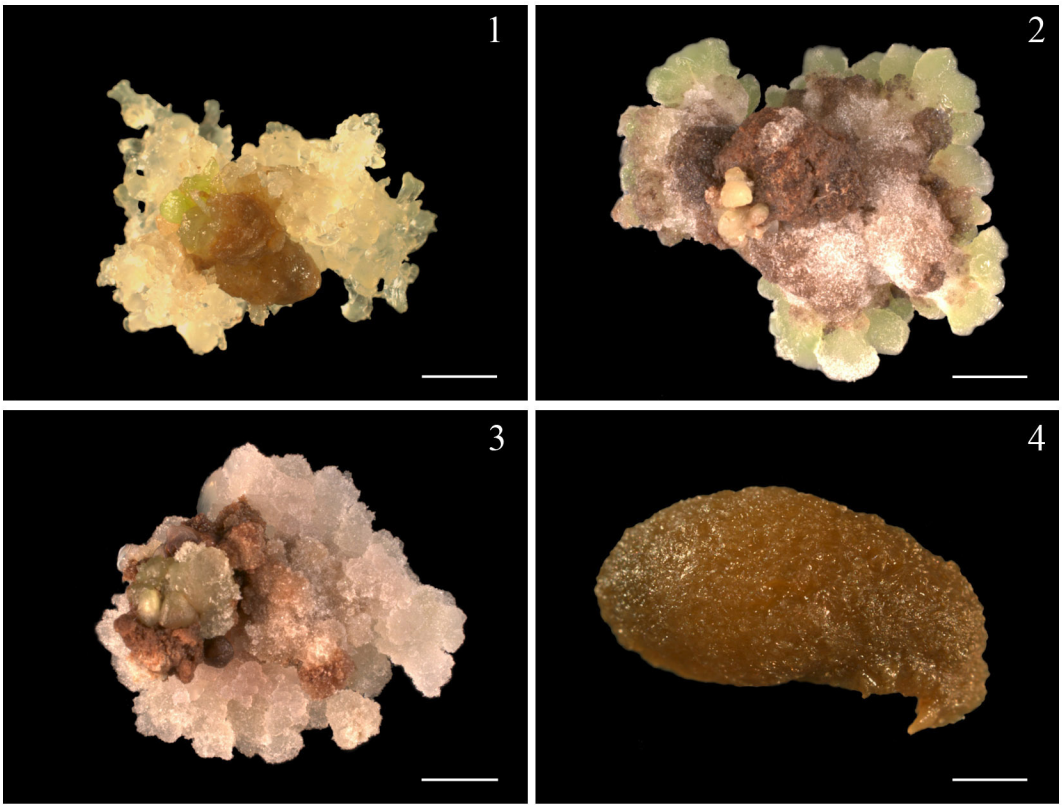
12 %. The open-pollinated crosses had the least successful induction rates. Their mean induction rate was 2 %. The induction rates for this group ranged from 1 % to 6 %.

During the induction process, 4.6 % of the zygotic embryos and megagametophytes used as explants were lost due to fungal or bacterial contamination. The induction rates presented in the tables only include *uncontaminated* explants. After the induction stage, no genotypes were lost to contamination.

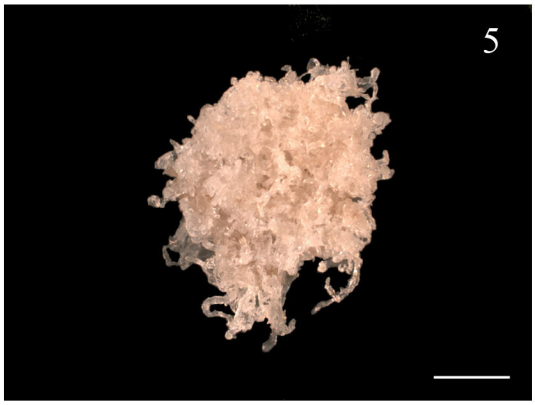
I observed multiple responses to the induction treatment by different genotypes (Figures 1 - 4). Some genotypes produced embryogenic cultures consisting of translucent immature embryos and suspensor cells (Figure 1). Other genotypes produced green or white non-embryogenic callus (Figures 2, 3). Non-embryogenic callus was incapable of producing somatic embryos, although, it appeared capable of growing indefinitely. I kept non-embryogenic callus growing for more than one year without any apparent loss in growth rate. Over this time, I did not observe any sign of organogenesis in any of the genotypes that produced non-embryogenic callus. Most commonly, explants did not respond to induction medium. In unresponsive genotypes, the zygotic embryo or megagametophyte explant eventually became necrotic (Figure 4).

Differences in the appearance of somatic embryo cultures were observed. Some cultures had loose aggregations of immature embryos (Figure 5), whereas others were very fine and dense (Figure 6). Some cultures were composed of both living immature embryos and necrotic embryos growing alongside one another (Figure 7). Cultures also varied in colour. Genotypes were varying shades of white, yellow and/or pink. Cultures

**Figures 1 - 4. Responses of Sitka spruce genotypes to induction treatment.** (1) Successfully induced zygotic embryo explant with translucent aggregations of immature somatic embryos (bar = 2 mm). (2) Non-embryogenic green callus (bar = 5 mm). (3) Non-embryogenic white callus (bar = 5 mm). (4) Necrotic megagametophyte explant (bar = 1 mm).


1
2
3
4

**Figures 5 – 7. Three established somatic embryogenic cultures, each with a different appearance.** (5) Loose aggregations of immature somatic embryos (bar = 5 mm). (6) Dense aggregations of immature somatic embryos (bar = 5 mm). (7) Aggregations of mixed embryogenic and necrotic immature somatic embryos (bar = 5 mm).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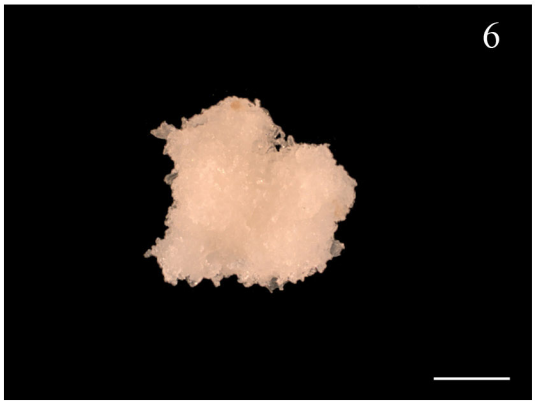
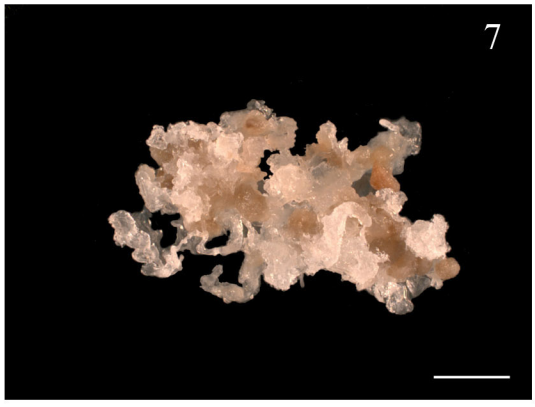

6


7

also differed noticeably in growth rates. Some cultures became necrotic within two weeks. Other cultures remained white with no necrosis.

**Maturation**

After embryogenic genotypes (n = 135) were placed onto maturation media, 88.1 % formed mature embryos (Table 4). This total includes all genotypes that produced at least one mature somatic embryo. The number of embryos per culture varied markedly. Responses of cultures to the maturation treatment were classified into four groups: 0 embryos per plate; < 10 embryos per plate; between 10 and 30 embryos per plate; > 30 embryos per plate (Table 4).

Overall, 60 % of genotypes showed a consistent response to the maturation treatment (Table 4). Consistency, in this case, refers to the embryo yield. Within this group, 26 % of genotypes produced more than 30 mature embryos per plate. About 27 % of the genotypes consistently produced between 10 and 30 embryos per plate. Similarly, 27 % of genotypes produced less than 10 embryos per plate. Lastly, 20 % of genotypes consistently failed to produce embryos on any plate (Table 4).

There were some genotypes that were less consistent, and varied in embryo yield from plate to plate. I observed a slightly variable response to the maturation treatment in 27 % of all genotypes (Table 4). This means that two different responses were observed on separate plates of a given genotype, but these responses were from neighbouring categories. Within this group, 46 % had some plates that produced greater than 30 embryos, and other plates that produced between 10 and 30 embryos. About 38 % of genotypes had plates that produced between 10 and 30 embryos, and other plates that produced less than 10 embryos. I found that 16 % of genotypes produced less than

**Table 4. Responses of individual genotypes to maturation treatment.** Each plate was screened for the approximate number of mature embryos per plate. The four possible response categories were: > 30 embryos per plate; between 10 and 30 embryos per plate; < 10 embryos per plate; 0 embryos per plate. Plus (+) signs represent an observed response in a given category. Minus (-) signs represent no observed response in a given category.

**Table 4**

| Genotype | Number of Plates | >30 | 10 to 30 | <10 | 0 |
|---|---|---|---|---|---|
| **Consistent** | | | | | |
| AB2 | 3 | + | - | - | - |
| AB8 | 3 | + | - | - | - |
| DB6 | 3 | + | - | - | - |
| DB8 | 3 | + | - | - | - |
| F2 | 3 | + | - | - | - |
| I3 | 3 | + | - | - | - |
| I4 | 3 | + | - | - | - |
| I6 | 3 | + | - | - | - |
| IL10 | 1 | + | - | - | - |
| IL11 | 3 | + | - | - | - |
| IL13 | 3 | + | - | - | - |
| IL19 | 3 | + | - | - | - |
| IL26 | 3 | + | - | - | - |
| IL32 | 3 | + | - | - | - |
| MN5 | 3 | + | - | - | - |
| MN8 | 3 | + | - | - | - |
| MN11 | 3 | + | - | - | - |
| MN15 | 1 | + | - | - | - |
| OI2 | 3 | + | - | - | - |
| OI9 | 3 | + | - | - | - |
| OL7 | 6 | + | - | - | - |
| AB4 | 1 | - | + | - | - |
| DB1 | 3 | - | + | - | - |
| EF1 | 1 | - | + | - | - |
| EF6 | 3 | - | + | - | - |
| FB1 | 3 | - | + | - | - |
| FB5 | 3 | - | + | - | - |
| GF4 | 3 | - | + | - | - |
| I10 | 3 | - | + | - | - |
| IL17 | 1 | - | + | - | - |
| IL23 | 3 | - | + | - | - |
| IL28 | 3 | - | + | - | - |
| IL38 | 3 | - | + | - | - |
| MN1 | 3 | - | + | - | - |
| MN17 | 1 | - | + | - | - |
| MN6 | 1 | - | + | - | - |
| OI5 | 3 | - | + | - | - |
| OL2 | 3 | - | + | - | - |
| OL3 | 3 | - | + | - | - |
| OL5 | 3 | - | + | - | - |
| TR11 | 3 | - | + | - | - |
| TS1 | 1 | - | + | - | - |
| TS3 | 1 | - | + | - | - |
| B3 | 1 | - | - | + | - |
| DA2 | 1 | - | - | + | - |

| Genotype | Number of Plates | >30 | 10 to 30 | <10 | 0 |
|---|---|---|---|---|---|
| DB3 | 1 | - | - | + | - |
| DB4 | 1 | - | - | + | - |
| DF6 | 1 | - | - | + | - |
| DF8 | 3 | - | - | + | - |
| EF2 | 1 | - | - | + | - |
| EF3 | 1 | - | - | + | - |
| FG2 | 1 | - | - | + | - |
| FG3 | 1 | - | - | + | - |
| KL1 | 1 | - | - | + | - |
| KL5 | 1 | - | - | + | - |
| KL7 | 1 | - | - | + | - |
| MN10 | 1 | - | - | + | - |
| NF4 | 1 | - | - | + | - |
| NF7 | 1 | - | - | + | - |
| NF8 | 1 | - | - | + | - |
| QR 4 | 1 | - | - | + | - |
| SR1 | 1 | - | - | + | - |
| SR6 | 1 | - | - | + | - |
| TR13 | 1 | - | - | + | - |
| TR5 | 1 | - | - | + | - |
| EF8 | 1 | - | - | - | + |
| FG1 | 3 | - | - | - | + |
| I7 | 3 | - | - | - | + |
| IL1 | 3 | - | - | - | + |
| IL20 | 3 | - | - | - | + |
| IL21 | 3 | - | - | - | + |
| IL22 | 3 | - | - | - | + |
| IL3 | 3 | - | - | - | + |
| MN16 | 1 | - | - | - | + |
| NF5 | 1 | - | - | - | + |
| QR1 | 3 | - | - | - | + |
| TR2 | 1 | - | - | - | + |
| TR3 | 3 | - | - | - | + |
| TR4 | 3 | - | - | - | + |
| TR6 | 1 | - | - | - | + |
| TR7 | 1 | - | - | - | + |
| **Slightly Variable** | | | | | |
| DB9 | 4 | + | + | - | - |
| FG5 | 3 | + | + | - | - |
| I2 | 3 | + | + | - | - |
| IL25 | 3 | + | + | - | - |
| IL31 | 3 | + | + | - | - |
| IL34 | 3 | + | + | - | - |
| IL5 | 3 | + | + | - | - |
| MF4 | 3 | + | + | - | - |
| MN2 | 3 | + | + | - | - |

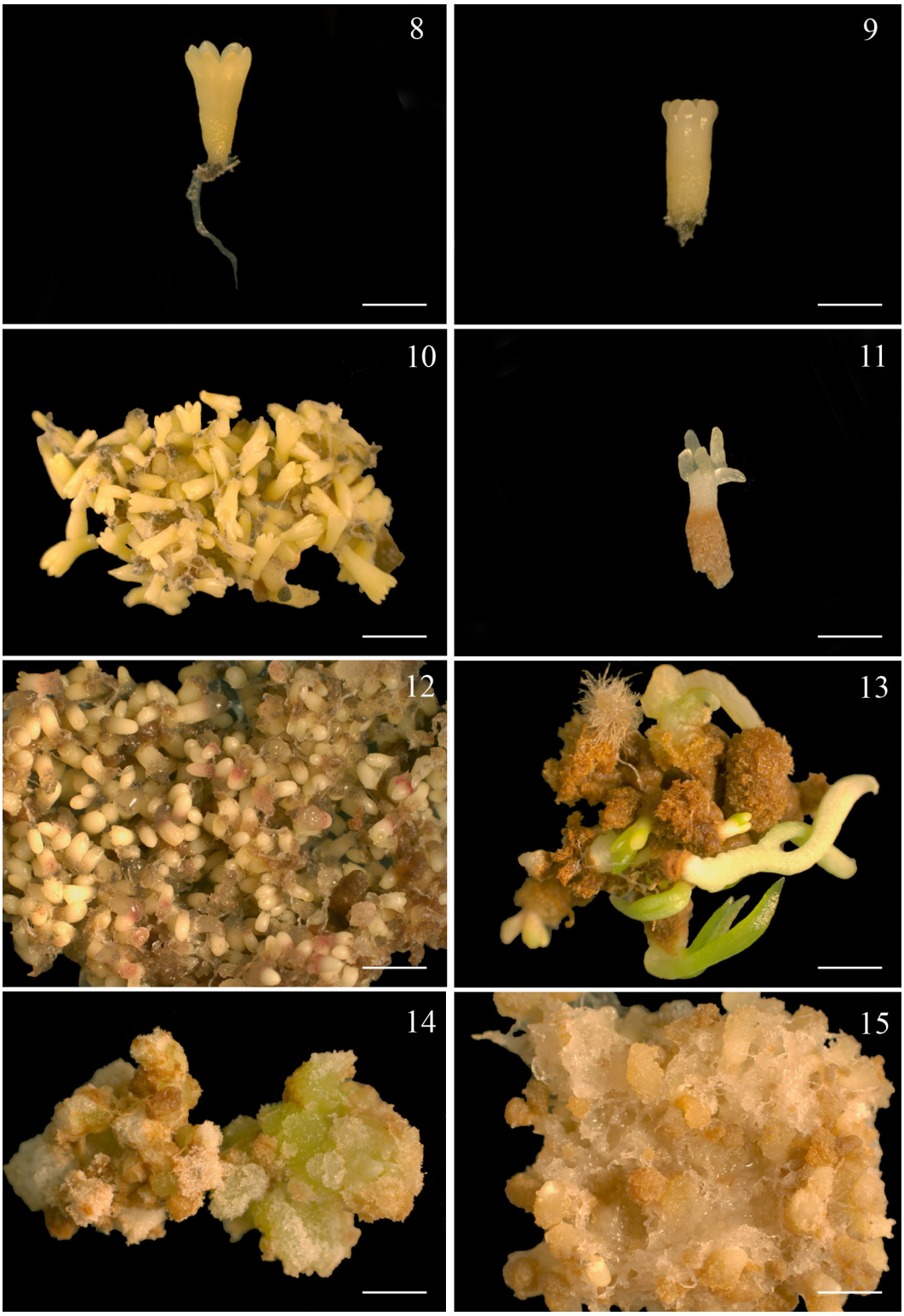
| Genotype | Number of Plates | >30 | 10 to 30 | <10 | 0 |
|---|---|---|---|---|---|
| NF1 | 3 | + | + | - | - |
| NF2 | 3 | + | + | - | - |
| NF3 | 3 | + | + | - | - |
| OL11 | 3 | + | + | - | - |
| OL13 | 3 | + | + | - | - |
| OL6 | 6 | + | + | - | - |
| SR10 | 3 | + | + | - | - |
| SR5 | 3 | + | + | - | - |
| AB6 | 3 | - | + | + | - |
| B1 | 3 | - | + | + | - |
| B2 | 2 | - | + | + | - |
| BC1 | 3 | - | + | + | - |
| GF2 | 3 | - | + | + | - |
| I1 | 3 | - | + | + | - |
| IL14 | 3 | - | + | + | - |
| IL29 | 3 | - | + | + | - |
| M1 | 3 | - | + | + | - |
| MN14 | 3 | - | + | + | - |
| OI1 | 3 | - | + | + | - |
| OI6 | 3 | - | + | + | - |
| SR2 | 3 | - | + | + | - |
| SR4 | 3 | - | + | + | - |
| AB5 | 3 | - | - | + | + |
| EF7 | 3 | - | - | + | + |
| EF11 | 3 | - | - | + | + |
| IL37 | 3 | - | - | + | + |
| JK2 | 3 | - | - | + | + |
| KL8 | 3 | - | - | + | + |
| **Highly Variable** | | | | | |
| IL36 | 3 | + | - | + | - |
| DF9 | 3 | - | + | - | + |
| EF5 | 3 | - | + | - | + |
| IL6 | 3 | - | + | - | + |
| IL15 | 3 | - | + | - | + |
| IL16 | 3 | - | + | - | + |
| KI1 | 3 | - | + | - | + |
| DB2 | 3 | + | - | - | + |
| IL18 | 3 | + | - | - | + |
| IL35 | 3 | + | - | - | + |
| KL2 | 3 | + | - | - | + |
| IL2 | 3 | + | + | + | - |
| KL3 | 3 | + | + | + | - |
| OI10 | 3 | + | + | + | - |
| IL24 | 3 | + | + | - | + |
| IL27 | 3 | + | + | - | + |
| OI3 | 3 | + | + | - | + |

10 embryos on some plates but no embryos on other plates (Table 4).

Some genotypes (13 %) had a completely inconsistent response to the maturation treatment (Table 4). Four of these genotypes had at least one plate that produced more than 30 mature cotyledonary embryos and at least one plate that produced no embryos. Three genotypes had one plate that produced more than 30 embryos, one plate that produced between 10 and 30 embryos, and one plate that produced less than 10 embryos. Another three genotypes had one plate that produced greater than 30 embryos, one plate that produced between 10 and 30 embryos, and one plate that failed to produce any embryos (Table 4).

Embryos from different genotypes showed diverse morphological responses to the maturation treatment. Many genotypes produced good quality embryos. These were opaque embryos with regularly-spaced cotyledons. Morphological differences in size and shape were often observed both within and between genotypes (Figures 8 and 9). Some embryos were narrow at the root cap and flared evenly to the cotyledons (Figure 8). Other genotypes were cylindrical in shape, with many tiny cotyledons (Figure 9). Embryos often varied in size and in number of cotyledons, ranging from 1 – 15. This variation occurred within the same aggregation of embryos (Figure 10). Some genotypes produced embryos with an irregular morphology. These had deformed cotyledons and appeared vitrified (Figure 11). Others were large and yellow, and failed to develop cotyledons (Figure 12). Yet other genotypes produced non-cotyledonary embryos with precociously germinated roots. These lacked epicotyls, or shoots. Another bizarre type produced only precocious shoots (Figure 13). These were all in addition to dead-ends, such as green or white non-embryogenic callus. These were identical to the callus types

**Figures 8 - 15. Morphological responses to maturation treatment.** (8) Good quality mature somatic embryo with an opaque yellow colour and regular cotyledons (bar = 1mm). (9) Good quality mature embryo with many small cotyledons (bar = 1 mm). (10) An aggregation of mature somatic embryos (bar = 2 mm). (11) Somatic embryo with deformed cotyledons and vitrified appearance (bar = 1 mm). (12) Somatic embryos that did not develop cotyledons (bar = 3 mm). (13) Culture producing precocious shoots (bar = 2 mm). (14) Green and white callus growth (bar = 3 mm). (15) Culture with continued embryogenic proliferation (bar = 3 mm).


8
9
10
11
12
13
14
15

occasionally formed from zygotic embryo explants during induction (Figure 14). Lastly, there were genotypes that simply continued to proliferate embryogenic tissue until they died, never having shown any inclination to differentiate (Figure 15).

**Germination**

Of the 45 genotypes placed onto germination medium, 44 germinated (Figure 16). The mean germination rate, referring to the *proportion of embryos* that germinated per genotype, was 80 % (Appendix 1). With the exception of one genotype that failed to germinate, the range for germination rate was between 35 and 100 % (Figure 16).

Germinants varied in quality and appearance. Some germinants had regular morphological features. They had a shoot that developed primary leaves, an elongated hypocotyl and a clearly distinguishable root (Figures 17 and 18). Shoots differed in size and shape. Leaves varied; some were longer or thicker than others. Roots sometimes reached several centimeters in length. Other roots were short. Some roots were white with many visible root hairs. Others were a shade of light brown without visible root hairs.

Germinants also varied in the regularity of their morphological features (Figures 19 - 21). Some were vitrified, with a swollen and glassy appearance (Figure 19). The base of the hypocotyl was often thicker on these germinants. They lacked clearly distinguishable roots. Other germinants had a curved hypocotyl, so much so that root and shoot were growing parallel to one another (Figure 20). The shoots of many germinants were also irregular, having deformed cotyledons and leaves. Sometimes the leaves were even forked (Figure 21). This variation in appearance was seen even among germinants

**Figure 16. Germination rates (%) of somatic embryos by genotype.** Between 15 and 48 embryos per genotype were placed on germination medium.

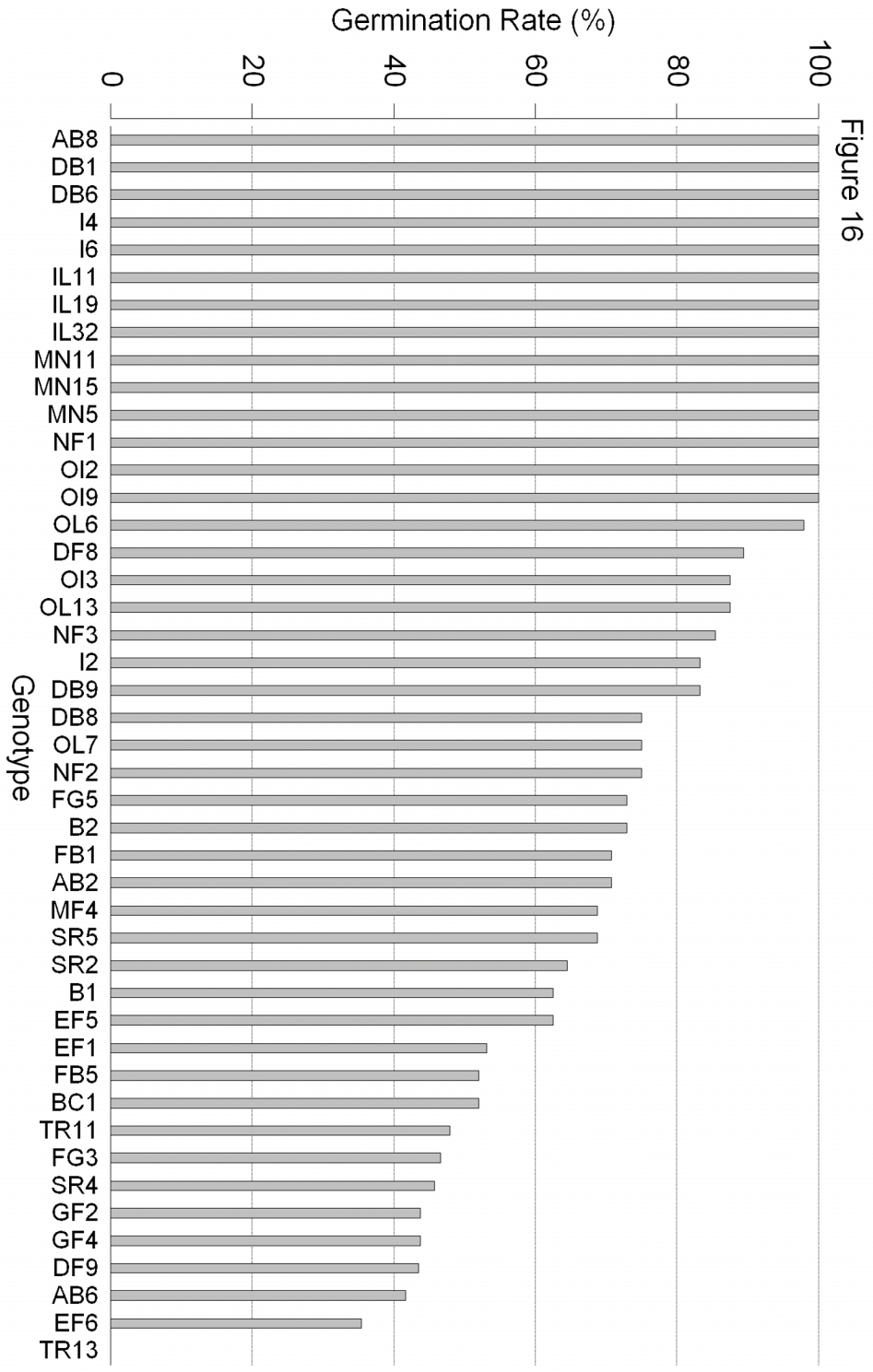

Germination Rate (%)
0
20
40
60
80
100
Genotype
AB8
DB1
DB6
I4
I6
IL11
IL19
IL32
MN11
MN15
MN5
NF1
OI2
OI9
OL6
DF8
OI3
OL13
NF3
I2
DB9
DB8
OL7
NF2
FG5
B2
FB1
AB2
MF4
SR5
SR2
B1
EF5
EF1
FB5
BC1
TR11
FG3
SR4
GF2
GF4
DF9
AB6
EF6
TR13

Figure 16

**Figures 17 - 21. Appearance of somatic germinants and seedlings**. (17) Good quality somatic germinant with shoot, straight hypocotyl and a longer root. (18) Good quality germinant with a shorter root. (19) Somatic germinant with vitrified appearance, swollen hypocotyl and no visible root. (20) Somatic germinant with curved hypocotyl. (21) Somatic germinant with a forked cotyledon. (Bar = 5 mm).

17

18

19

20

21

of a given genotype. Often a single germination plate would contain some germinants that were considered to be of good quality and some that were deformed in one or more ways.

**Acclimatization and Growth**

All 45 genotypes that were placed on germination medium were transferred to *ex vitro* conditions. Only 5.5 % of germinants were successfully converted to seedlings (Appendix 2). The conversion rates ranged from 0 % to 33 % over the different genotypes (Figure 22). About half (23 of 45) of the genotypes were able to convert. Six of these genotypes had a conversion rate greater than 10 % and 17 of these genotypes had conversion rates less than or equal to 10 %. This means that all of the germinants from the remaining 22 genotypes died (Figure 22). Most germinants died as a result of a fungus gnat infection.

One genotype (TR13) that did not germinate eventually produced seven shoots organogenically. All of these were transferred to *ex vitro* conditions but died soon after.

Seedlings each had an orthotropic shoot with branches (Figure 23). Needles were soft and bright green in colour for the first few months after being transferred to soil. With time, the needles darkened and became more rigid. Many seedlings set bud when the photoperiod decreased in October 2009. These seedlings broke bud and resumed growth once artificial light was applied. Most seedlings developed root systems that had reached the bottom of the styroblock by October 2009.

**Figure 22. Rates of conversion (%) from somatic germinant to somatic seedling by genotype.**

Figure 22

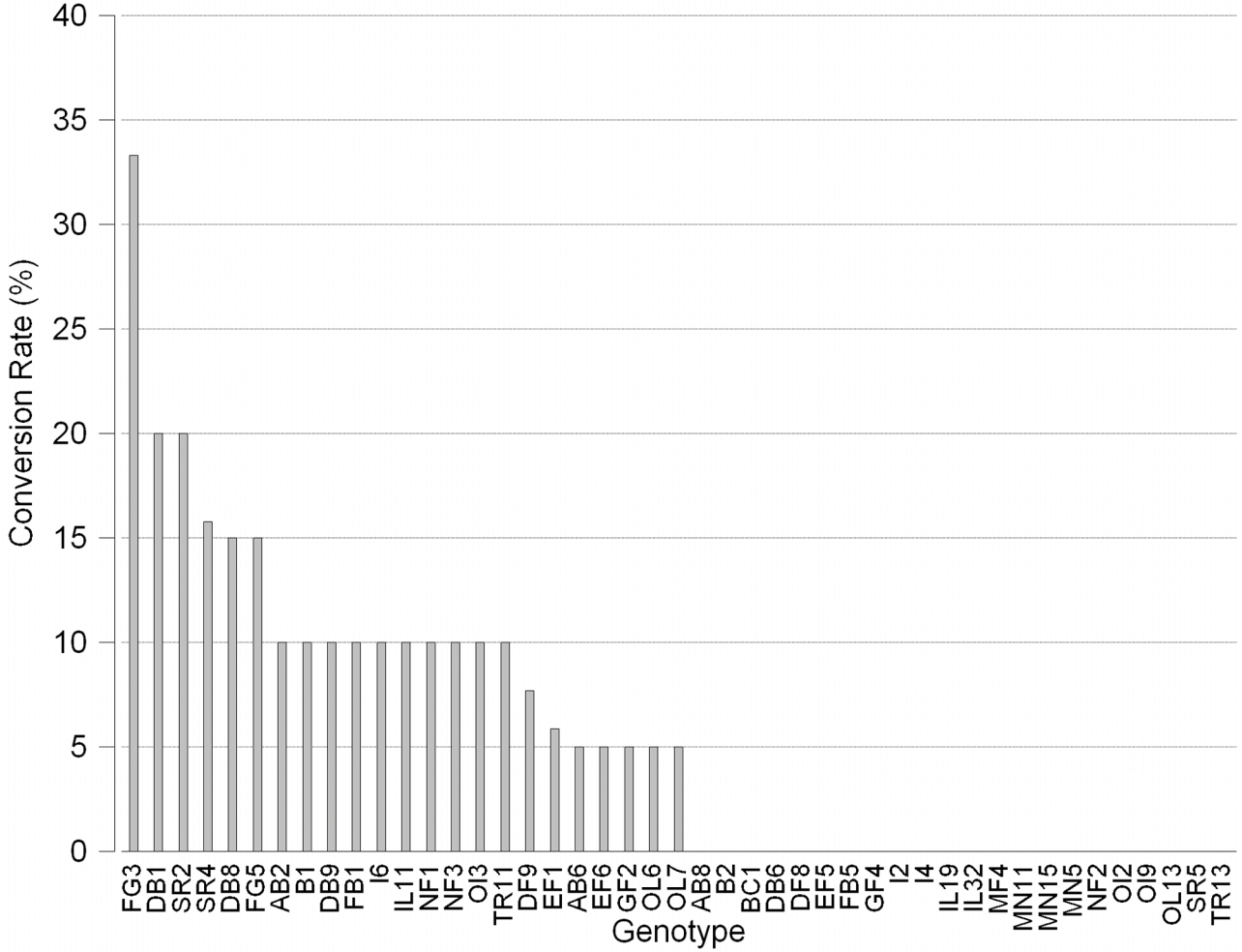

Conversion Rate (%)
40
35
30
25
20
15
10
5
0
FG3
DB1
SR2
SR4
DB8
FG5
AB2
B1
DB9
FB1
I6
IL11
NF1
NF3
OI3
TR11
DF9
EF1
AB6
EF6
GF2
OL6
OL7
AB8
B2
BC1
DB6
DF8
EF5
FB5
GF4
I2
I4
IL19
IL32
MF4
MN11
MN15
MN5
NF2
OI2
OI9
OL13
SR5
TR13
Genotype

**Figure 23. Somatic seedling of Sitka spruce** (bar = 2 cm)**.**


23

**Cryopreservation**

Pretreatment

A total of 112 genotypes (cultures of immature embryos) were placed onto modified maturation medium and kept at 5 °C as a pretreatment step before cryopreservation. I observed four different responses to this 5 °C pretreatment. In some genotypes, immature embryos were all converted to mature yellow embryos, some of which had cotyledons (Figure 24). Other genotypes produced many maturing embryos, but it was obvious that many remained immature (Figure 25). Other genotypes showed little response to the pretreatment at 5 °C. These genotypes had few maturing embryos per plate (Figure 26). There were also genotypes that did not respond to the pretreatment. These genotypes produced no mature embryos (Figure 27).

Tolerance to Freezing and Thawing

To determine the ideal duration for the 5 °C (chilling) pretreatment, genotypes were tested for their ability to survive freezing in liquid nitrogen and subsequent thawing at three time-points. After four weeks of chilling, five genotypes were tested. No lines survived. After eight weeks of the 5 °C pretreatment, nine genotypes were tested. Of these, two-thirds were capable of producing somatic embryos and a third of the lines developed into non-embryogenic callus. The last test was done after twelve weeks of chilling. I tested 103 genotypes. Only 28 % of the genotypes were able to produce somatic embryos. An additional 34 % survived, but these developed into non-embryogenic callus. The remaining 38 % of genotypes died.

I pooled the responses of all genotypes tested at the second and third time-points (Figure 28). Of the 112 genotypes tested, I was able to recover embryogenic cultures

**Figures 24 - 27. Response to 5 °C pretreatment before cryopreservation.** (24) Cultures completely filled with maturing yellow embryos. Some genotypes have embryos with cotyledons. (25) Cultures having many maturing embryos, but with some translucent immature embryos still visible. No embryos have cotyledons. (26) Cultures having very few maturing embryos. (27) Cultures having no maturing embryos. (All bars = 2 mm).

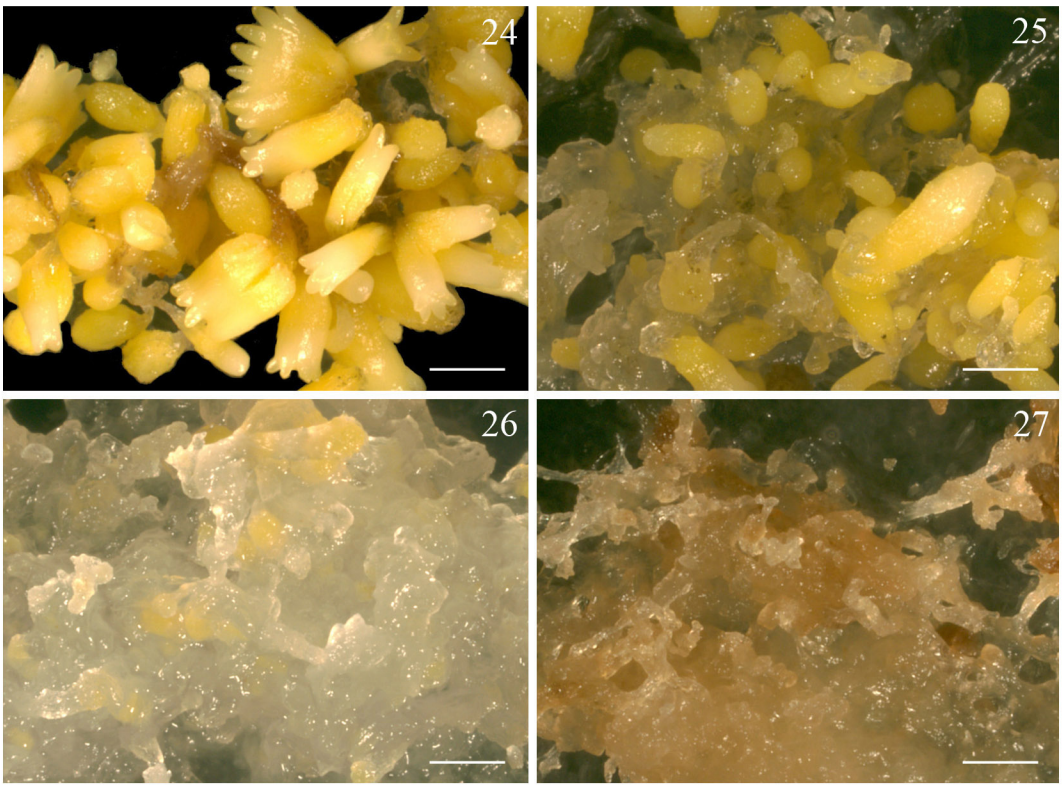
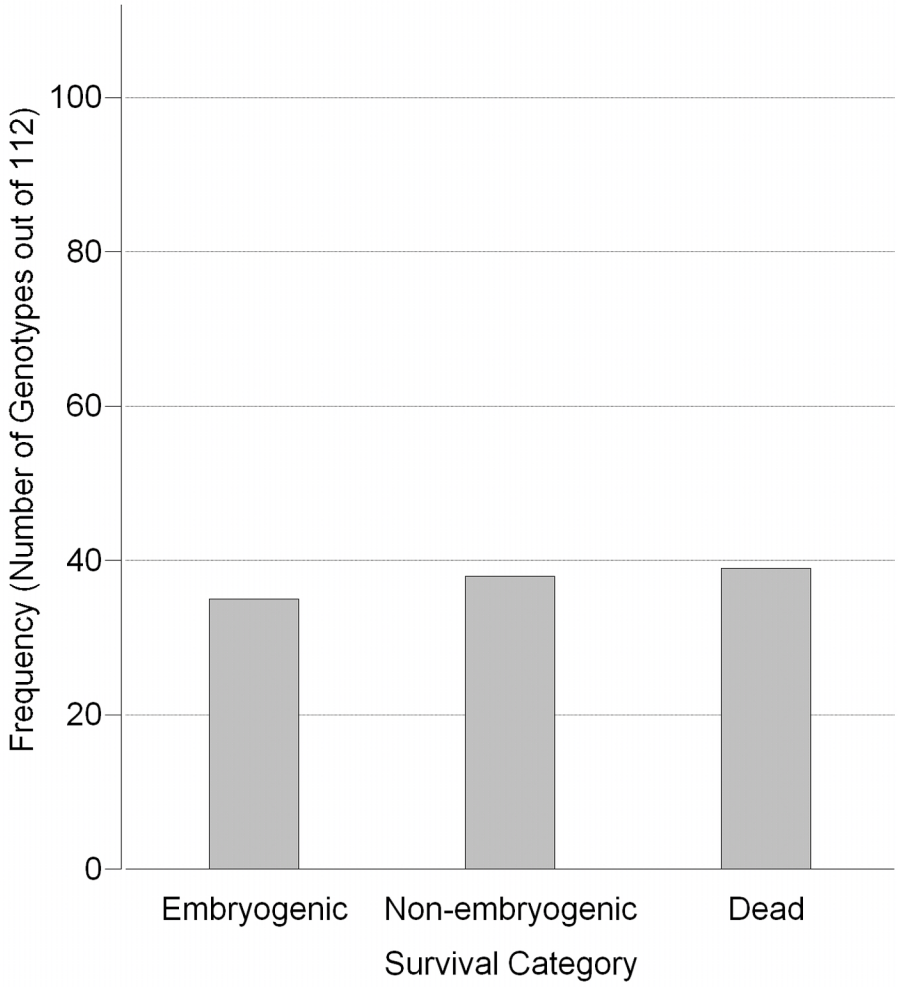

24
25
26
27

**Figure 28. Response of genotypes (n = 112) to cryopreservation.** Three possible responses were recorded: embryogenic, non-embryogenic, or dead. Frequency is given as the number of genotypes.

Figure 28


Frequency (Number of Genotypes out of 112)
100
80
60
40
20
0
Embryogenic
Non-embryogenic
Dead
Survival Category

from 31 % of genotypes. Another 34 % of genotypes survived, but produced non-embryogenic callus that was unable to produce somatic embryos. Many genotypes (35 %) did not survive the freezing and thawing (Figure 28).

Comparison of 5 °C Pretreatment and Tolerance to Freezing and Thawing

I compared the appearance of each genotype after the 5 °C pretreatment to its ability to survive cryopreservation (Table 5). The categories used to describe the appearance of genotypes after the pretreatment are those shown in Figures 24 - 27. I found that 25 of the 112 genotypes produced abundant maturing yellow embryos during the pretreatment, some with cotyledons. These genotypes were assigned a rank of 1. This group corresponds to Figure 24. Of the genotypes in this group, 68 % were able to produce embryogenic cultures after cryopreservation. The other 32 % of these genotypes produced non-embryogenic callus that could not develop into embryogenic cultures. None of these genotypes died after being frozen and thawed for cryopreservation.

The second group was made up of genotypes (54 of 112) that produced some maturing embryos during the pretreatment, but did not produce any embryos with cotyledons (Table 5). These genotypes were assigned the rank of 2. This group corresponds to Figure 25. These genotypes had a variable response to the cryopreservation treatment. About 31 % of these genotypes produced embryogenic cultures after being frozen and thawed. A greater proportion of these genotypes (41 %) produced non-embryogenic callus in response to the pretreatment. About 30 % of these genotypes died after the cryopreservation treatment.

Genotypes that produced no or a few maturing embryos during the pretreatment made up the last two groups (Table 5). These groups were assigned the ranks 3 and 4

**Table 5. Observed responses of genotypes (n=112) to 5 °C pretreatment and their survival responses to cryopreservation.** Genotypes were ranked according to the categories presented in Figures 24-27: (1) all immature embryos were converted to mature yellow embryos, some with cotyledons; (2) many mature embryos developed, but many remained immature; (3) culture produced few mature embryos; (4) culture produced no mature embryos. After cryopreservation, the response of each genotype was recorded. Genotypes produced embryogenic tissue, produced non-embryogenic callus or they died. The response was recorded as a plus (+) sign.

**Table 5.**

| Genotype | Rank (Plate 1) | Rank (Plate 2) | Embryogenic | Callus | Dead |
|---|---|---|---|---|---|
| B1 | 1 | 1 | + | | |
| BC1 | 1 | 1 | + | | |
| EF7 | 1 | 1 | + | | |
| FB1 | 1 | - | + | | |
| FG3 | 1 | 1 | + | | |
| GF2 | 1 | 1 | + | | |
| GF4 | 1 | 1 | + | | |
| I2 | 1 | 1 | + | | |
| I6 | 1 | 1 | + | | |
| IL2 | 1 | 1 | + | | |
| IL16 | 1 | - | + | | |
| IL13 | 1 | 1 | + | | |
| IL37 | 1 | 1 | + | | |
| KI1 | 1 | - | + | | |
| OL2 | 1 | 1 | + | | |
| NF1 | 1 | 1 | + | | |
| NF2 | 1 | 1 | + | | |
| B2* | 1 | 1 | | + | |
| I10 | 1 | 1 | | + | |
| IL18 | 1 | - | | + | |
| OI5 | 1 | 1 | | + | |
| DB8 | 1 | 2 | | + | |
| IL5 | 1 | 2 | | + | |
| IL10 | 2 | 1 | | + | |
| NF3 | 1 | 2 | | + | |
| AB2 | 2 | 2 | + | | |
| DB2 | 2 | 2 | + | | |
| DB9 | 2 | 2 | + | | |
| F2 | 2 | 2 | + | | |
| FB5 | 2 | 2 | + | | |
| IL26 | 2 | - | + | | |
| IL32 | 2 | 2 | + | | |
| KL1 | 2 | 2 | + | | |
| M1 | 2 | 2 | + | | |
| MN5 | 2 | 2 | + | | |
| MN8 | 2 | - | + | | |
| MN11 | 2 | 2 | + | | |
| OI2 | 2 | 2 | + | | |
| QR 4 | 2 | - | + | | |
| SR4 | 2 | 2 | + | | |
| SR5 | 2 | 2 | + | | |
| TS3 | 2 | 2 | + | | |
| EF11 | 2 | 2 | | + | |
| FG5 | 2 | 2 | | + | |
| I3 | 2 | 2 | | + | |
| IL14 | 2 | 2 | | + | |
| IL19 | 2 | 2 | | + | |
| IL21 | 2 | 2 | | + | |
| IL24 | 2 | 2 | | + | |
| IL29 | 2 | 2 | | + | |
| IL31 | 2 | 2 | | + | |
| IL36 | 2 | 2 | | + | |
| KL2 | 2 | 2 | | + | |
| KL3 | 2 | 2 | | + | |
| MN17 | 2 | - | | + | |
| OL11 | 2 | 2 | | + | |
| OL13 | 2 | 2 | | + | |
| OL6 | 2 | 2 | | + | |
| OL7 | 2 | 2 | | + | |
| QR1 | 2 | 2 | | + | |
| SR1 | 2 | 2 | | + | |
| TR5 | 2 | - | | + | |
| TR13 | 2 | 2 | | + | |
| AB8 | 2 | 2 | | | + |
| DB1 | 2 | 2 | | | + |
| EF3 | 2 | 2 | | | + |
| EF6 | 2 | 2 | | | + |
| FG1 | 2 | 2 | | | + |
| IL11 | 2 | 2 | | | + |
| IL23 | 2 | 2 | | | + |
| IL28 | 2 | 2 | | | + |
| MF4 | 2 | 2 | | | + |
| OI1 | 2 | 2 | | | + |
| OI9 | 2 | 2 | | | + |
| SR2 | 2 | 2 | | | + |
| SR10 | 2 | 2 | | | + |
| IL34 | 3 | 2 | | + | |
| IL25 | 2 | 3 | | | + |
| OI10 | 2 | 3 | | | + |
| AB5 | 3 | 3 | + | | |
| AB4 | 3 | 3 | | + | |
| DF6 | 3 | 3 | | + | |
| I1 | 3 | 3 | | + | |
| MN10 | 3 | 3 | | + | |
| MN14 | 3 | 3 | | + | |
| OL5 | 3 | 3 | | + | |
| TR11 | 3 | 3 | | + | |
| AB6 | 3 | 3 | | | + |
| DA2 | 3 | 3 | | | + |
| DB4 | 3 | 3 | | | + |
| DB6 | 3 | 3 | | | + |
| DF8 | 3 | 3 | | | + |
| DF9 | 3 | 3 | | | + |
| EF5 | 3 | 3 | | | + |
| I4 | 3 | 3 | | | + |
| IL17 | 3 | 3 | | | + |
| IL22 | 3 | 3 | | | + |
| IL35 | 3 | 3 | | | + |
| KL5 | 3 | 3 | | | + |
| KL8 | 3 | 3 | | | + |
| MN1 | 3 | 3 | | | + |
| MN6 | 3 | 3 | | | + |
| OI6 | 3 | 3 | | | + |
| OL3 | 3 | 3 | | | + |
| IL15 | 4 | 3 | | + | |
| EF1 | 3 | 4 | | | + |
| IL6 | 3 | 4 | | | + |
| MN15 | 3 | 4 | | | + |
| B3 | 4 | 4 | | | + |
| DB3 | 4 | 4 | | | + |
| EF2 | 4 | 4 | | | + |
| IL38 | 4 | 4 | | | + |

respectively. These genotypes corresponded to Figures 26 and 27. This group constituted 33 of 112 genotypes. Their ability to survive freezing and thawing varied slightly. Only one of these 33 genotypes produced embryogenic cultures after thawing. About 24 % of these genotypes produced non-embryogenic callus. The remaining 73 % of these genotypes died after the cryopreservation process.

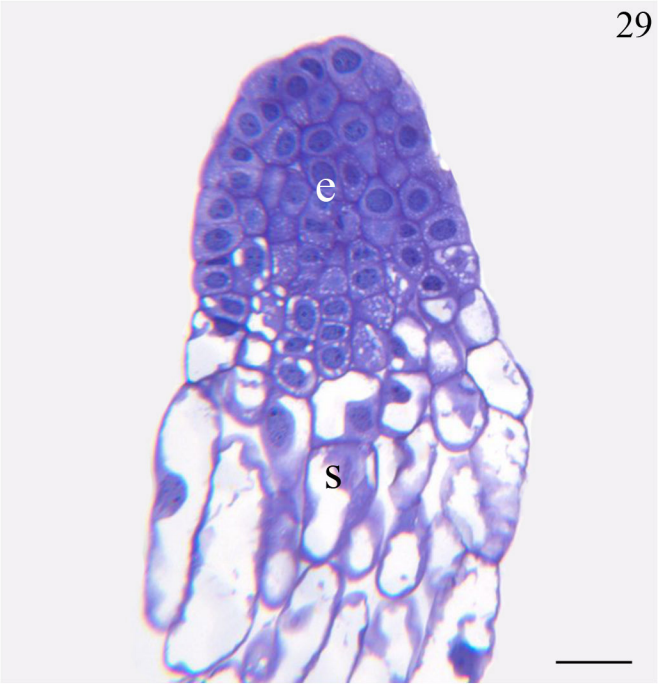
**Developmental Series**

Four stages of development were sectioned. The early somatic embryo consisted of an embryonal mass, composed of densely stained, cytoplasmically rich cells, and a suspensor, made up of large, elongated, vacuolated cells (Figure 29).

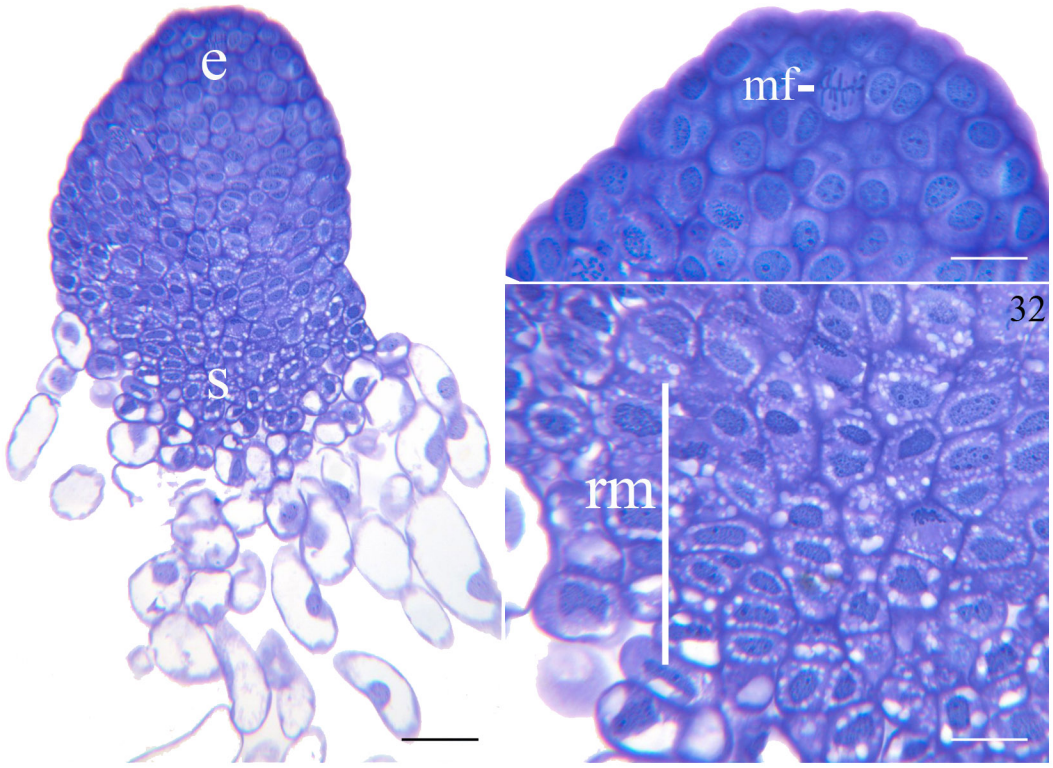
Somatic embryos then became globular in shape (Figure 30). Cells of the embryonal mass were restricted to the apical portion. These stained very darkly at this stage as their cytoplasms were quite dense. Within the embryonal mass, isodiametrically shaped cells at the apical end were slightly smaller and more densely stained than those nearer to the distal end, where cells were larger and had visible vacuoles. Mitotic figures indicated that cells were actively dividing throughout the embryonal mass (Figure 31). A rib meristem was visible near the distal end of the embryo (Figure 32). The rib meristem added bulk to the embryo by producing suspensor cells in one direction and embryonal cells in the other. The division between the embryonal mass and suspensor was distinct.

The embryos developed a cylindrical shape with a flattened apical end (Figure 33). Layers of tissue became visible in the hypocotyl. Larger, rectangular cells made up a pith ground meristem (Figure 34). Smaller and more rectangular cells made up the procambium, while larger and rounder cells formed the cortical ground meristem. A

**Figure 29. Early Sitka spruce somatic embryo.** The darkly stained embryonal mass (e) is distinct from the suspensor (s) (bar = 50 μm).


29
e
s

**Figures 30 – 32. Globular stage of Sitka spruce somatic embryo**. (30) Globular somatic embryo with darkly stained embryonal mass (e) and vacuolated suspensor (s) (bar = 100 μm). (31) Mitotic figure (mf) (bar = 50 μm). (32) Rib meristem (rm) (bar = 40 μm).


30
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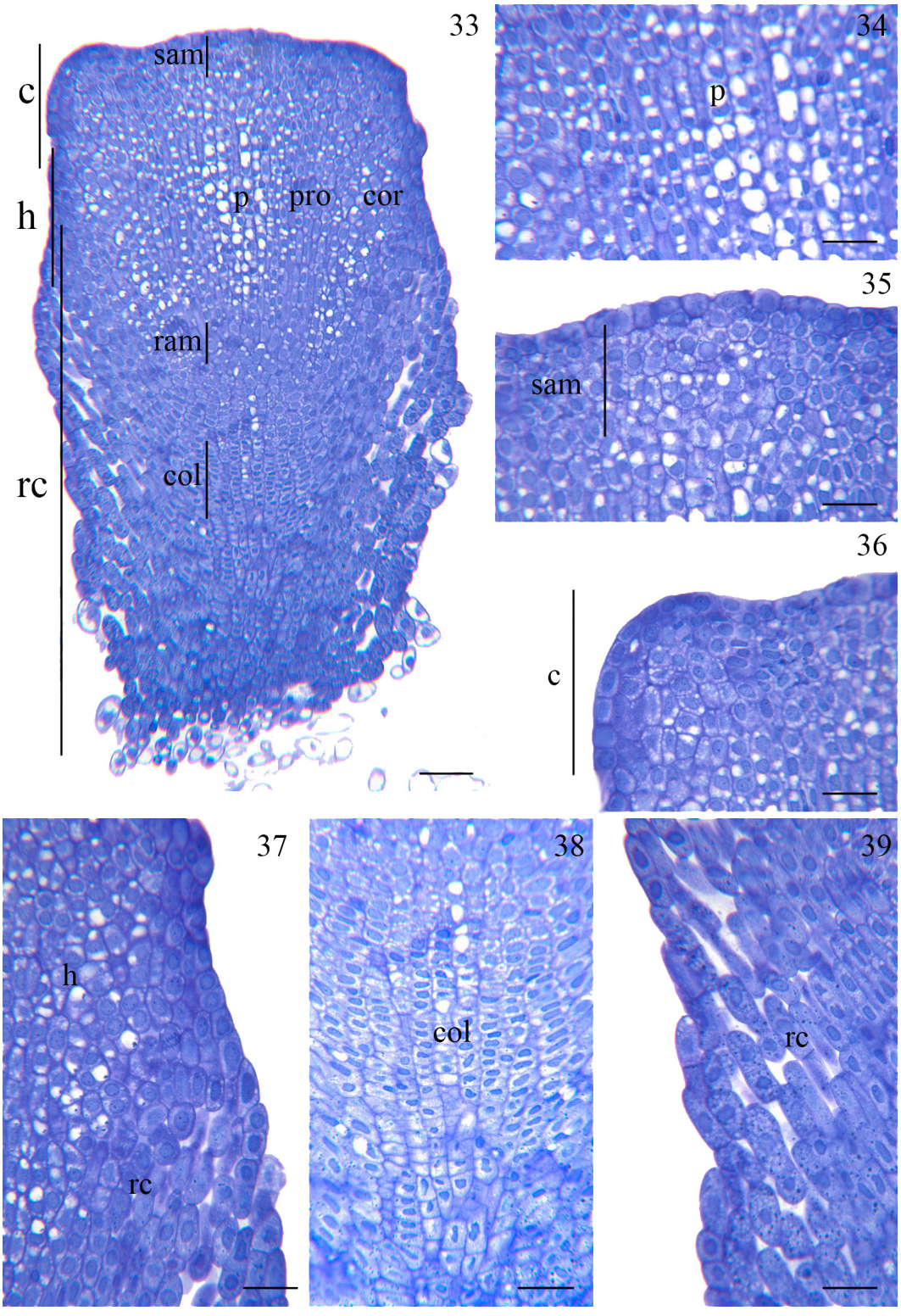
**Figures 33 – 39. Somatic embryo with flattened apical end.** (33) Whole somatic embryo, showing developing cotyledon (c), hypocotyl (h), root cap (rc), root apical meristem (ram), pith (p), cortex (cor), procambium (pro) (bar = 100 µm). (34) Detail of the pith (p) (bar = 50 µm). (35) Shoot apical meristem (sam) with large isodiametic cells (bar = 50 µm). (36) Developing cotyledon (c) (bar = 50 µm). (37) Detail of transition between hypocotyl (h) and root cap (rc) (bar = 50 µm). (38) Column cells (col) (bar = 50 µm). (39) Loosely packed cells of the root cap (rc) (bar = 50 µm).


33
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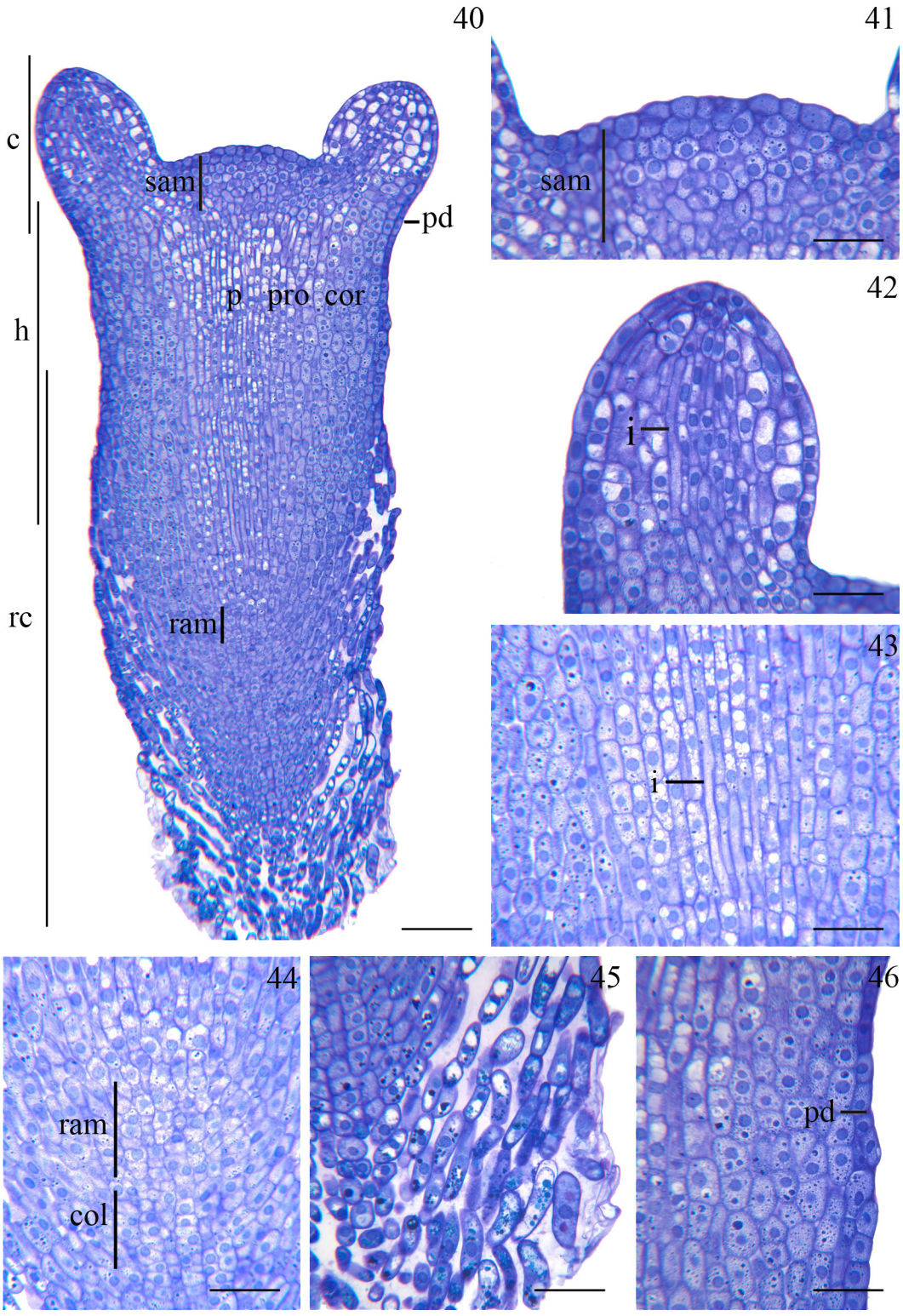
38
col
39
rc

shoot apical meristem, consisting of large isodiametric cytoplasmically rich cells was visible (Figure 35). Cotyledons could be seen developing as rounded projections from the edge of the flattened apical end (Figure 36). Protoderm was visible as regularly-sized cells around the edge of the embryo. The transition between hypocotyl and root was gradual (Figure 37). The root meristem formed before the shoot apical meristem. The root cap consisted of two regions: large, loosely packed cells at the outside and columns of cells in the centre (Figure 38) near the root apical meristem. Root cap cells often contained deposits of polyphenols, indicated by green staining with Toluidine blue (Figure 39). Cell division was evident throughout the embryo, showing that embryo growth was not only due to cell expansion.

As the somatic embryos matured, growth slowed. Mature embryos had a longer and more developed hypocotyl, with clearly distinguished pith, procambium and cortex (Figure 40). The shoot meristem formed a dome-shaped structure at the apical end of the embryo (Figure 41). The cotyledons developed pith, procambium and cortex (Figure 42). Large, long idioblast cells were apparent within the cotyledons and hypocotyl, appearing both in the pith and the cortex (Figures 42 and 43). The root apical meristem was subtended by an extended column (Figure 44). The root cap enveloped the entire lower half of the embryo. Many large, loosely packed cells filled with polyphenol deposits were visible (Figure 45). The root cap connected to the protodermal cells (Figure 46) and hypocotyl of the upper half of the embryo.

**Figures 40 – 46. Mature Sitka spruce somatic embryo.** (40) Whole mature somatic embryo with developed cotyledons (c), hypocotyl (h), and root cap (rc). The pith (p), procambium (pr) and cortex (cor) of the hypocotyl are defined (bar = 200 µm). (41) Detail of the dome-shaped shoot apical meristem (sam) (bar = 100 µm). (42) Cotyledon with pith, cortex and procambium. An idioblast (i) is visible in the pith (bar = 100 µm). (43) Idioblast visible in the pith of the hypocotyl (bar = 100 µm). (44) Cells of the root apical meristem (ram) and column (col) (bar = 100 µm). (45) Root cap cells with polyphenol deposits (bar = 100 µm). (46) Detail of the protoderm (pd) cells of the hypocotyl (bar = 100 µm).


40
c
sam
pd
p
pro
cor
h
rc
ram
41
sam
42
i
43
i
44
ram
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pd

## Discussion

### Phenotypic and Genotypic Variation

Understanding weevil resistance in Sitka spruce is difficult, not only because of the tree's long-lived nature, but its wide range and genotypic diversity. To unravel resistance, it is best to break it down into parts. This somatic embryogenesis project is just one small piece of the larger puzzle concerning genotypic and phenotypic variation of weevil-resistance in Sitka spruce.

The BC Sitka spruce weevil-resistance breeding program exploited genotypic variation, thereby providing the basic genetic materials for this project. Over the past 30 years, this breeding program has worked to identify and to breed sources of resistant trees (King and Alfaro 2009). These programs involved initial extensive screening for resistant provenances. Subsequently, the geographic ranges of these resistant populations were defined. After intensive study, resistance mechanisms were identified and linked to specific genotypes. Amongst other biochemical and phenological traits, these mechanisms include three physical bark traits: constitutive resin canals, traumatic resin canals and sclereid cells (King and Alfaro 2009). The question to be answered is how consistent such traits are within a given genotype. The best way to answer this question is to use clones. Cloning exposes phenotypic variation by eliminating genotypic variation: identical ramets can be grown and their variability assessed.

One way to clone conifers is to induce somatic embryogenesis. This method has certain advantages. Specific genotypes can be cryo-stored for long periods of time during longer-term trials of immature and mature trees (Tautorus et al. 1991; Park et al. 1998). Somatic embryogenesis produces pest- and disease-free stock. It does not require

grafting stock to be held in orchards. It can also provide a fast method to bulk-up large numbers of individual genotypes (Tautorus et al. 1991).

My project was successful in taking seed material from the BC Sitka spruce breeding program and turning it into a system that could potentially be used to study phenotypic variation in weevil-resistance traits. I was able to establish the entire SE process. This demonstrates that an SE system can be set up using material selected for specific traits.

The overarching idea behind this project is to explore phenotypic variation. In conifers, this is seen in many traits, including wood quality (St. Clair 1994) and pest resistance (Wells et al. 1982; White and Nilsson 1984). From my research, it is also clear that phenotypic variation is evident from the earliest stage of conifer embryogenesis onwards. This project only includes the first year of growth in conifer seedlings, yet differences in phenotypes are obvious.

Phenotypic variation has also been observed in zygotic embryos. Evidence for variation during conifer embryogenesis has been found in a number of different species for various seed characteristics including seed size (Wyman et al. 1997), embryo size (Sorensen and Franklin 1977), and cotyledon number (Brian et al. 1998; Kuser and Ching 1981).

We can look at phenotypic variation from two perspectives: within genotypes and among genotypes. Phenotypic plasticity refers to the flexibility of one genotype to develop various phenotypes depending on its interactions with its environment (Miner et al. 2005). Genotypic variation explains the differences in phenotype seen over the range of a species. It accounts for differences between different genotypes of a species

interacting with their environments (Rehfeldt et al. 1999). Both of these types of phenotypic variation were apparent throughout this project.

Phenotypic plasticity in somatic embryo cultures has not gone unnoticed in the past. Von Aderkas (2002) reported variability in the number of cotyledons for somatic embryos of the same genotype. Harrison and von Aderkas (2004) showed that cotyledon development was controlled by the levels of plant growth regulators. It has also been shown that somatic embryos from the same genotype can be induced to have different freezing tolerances just by controlling the temperature at which they mature (von Aderkas et al. 2007). Phenotypic variation within genotype was evident throughout my project. A given genotype placed on maturation medium did not always produce the same response in replicates. Even within the same plate, embryos differed in size and number of cotyledons. Replicate plates exposed to the cryopreservation pretreatment sometimes showed a variable response.

Phenotypic plasticity in conifer clones deserves to be studied. Plasticity allows organisms to adjust to their environments (Sultan 2004). It appears that even subtle environmental differences, in this case within a Petri dish, can still cause phenotypic differences. Somatic embryogenesis can provide a system in which phenotypic plasticity can be explored.

The influence of genetics in the control over each stage of somatic embryogenesis has been investigated thoroughly by Park et al. (1993, 1994). They showed that genetic control over SE is high in the induction phase, and decreases for both maturation and germination. During each stage of this project, such genotypic differences were obvious. There were different responses to induction. Some genotypes produced embryogenic

tissue, while others produced callus or died. During maturation, some genotypes produced good quality embryos, while others did not mature. Some genotypes could germinate, while others could not. Even the appearance of the embryos and germinants differed between genotypes. It was clear in this project that genotype is an important factor in the SE process. Perhaps it was because I used a large number of genotypes that these differences became so obvious.

**Breeding**

The goal of this project was to create a system for studying phenotypic variation in resistance traits in Sitka spruce. The most ideal way to look at phenotypic variation is to look at the extremes of the trait. Differences are greatest between extremes. The Sitka spruce breeding program included not only genotypes with great abundances of these resistance traits, but also genotypes having unusually low levels of these traits (Grau 2006; King and Alfaro 2009). This allowed me to select crosses at both extremes for each trait. Knowing these traits are heritable (Wainhouse and Ashburner 1996; King et al. 2004), and being able to select extreme parent genotypes let me produce seeds more likely to have extreme phenotypes.

The starting material for this project came from the British Columbia Ministry of Forests weevil-resistance breeding program for Sitka spruce (King and Alfaro 2009). Each of the parental genotypes that I used had been characterized for three physical resistance traits: constitutive resin canals, traumatic resin canals and sclereid cells. Genotypes had been ranked separately for each of these traits. From the outset, using well-characterized parental genotypes added value to this system. It is a more efficient strategy to use known genotypes rather than looking for traits at random in a wild

population. Furthermore, applying tissue culture methods – with all their complexity and required skill – is only warranted when characterized and valuable genetic stocks are used. This approach let me focus my effort on material that is more likely to answer questions about these resistance traits in the resulting SE clonal lines.

Using controlled-pollinations, I selected for known resistance traits. If I had used open-pollinated material or poly-mix crosses, I would have been less certain of these resistance traits, since I would have only known one of the parental genotypes.

This strategy does have its limits. Due to recombination, there is no guarantee that the seeds produced from controlled-pollinations will show as extreme a phenotype as their parents. This means that some SE clonal lines derived from these seeds may not exhibit these desired extreme phenotypes. There is no way of knowing until the resulting seedlings can be grown and examined for phenotype. This is the limitation of using progeny to create clonal lines. Selected traits in progeny are only apparent once seedlings are fully grown, which means that there is an element of anticipation in this approach. A solution to this problem would be to clone tissue from characterized mature trees. Unfortunately, this is currently not possible in spite of many heroic attempts, not to mention fraudulent claims.

There are also practical difficulties in producing control-pollinated seed. This was evident in this project. Seed-set was low for the controlled-pollination crosses of Sitka spruce. On average, I found 9.4 ± 6.9 (mean ± SD) filled seeds per cone. By comparison, Sitka spruce has the potential to produce up to 284 seeds per cone (Owens and Molder 1980). Seed yield was not a problem with open-pollinated material in this project. I was easily able to extract 40 filled seeds per cone.

There are a number of possible causes for poor seed-set. Pollen quality is one of these (Bonnet-Masimbert and Webber 1995). All pollen was tested by Cathy Cook of Western Forest Products for electrical conductivity. Only pollen passing the set standards was used. The controlled-pollinations were conducted by Dr. John King and David Ponsford, both experts with more than 30 years of tree breeding experience. Timing of pollinations is another factor (Sorensen and Webber 1997). The female cones must be at the correct developmental stage for pollination to be successful. The date that this occurs each year is influenced by the weather patterns of that particular year. In 2008, we experienced an unusually late and rainy spring. Weather considerations are therefore one possible explanation. Also, the female cones were bagged to isolate them from undesired pollen. Bagging can create micro-climatic differences that speed up development of the female cones (Runions et al. 1995). This could have lessened the available number of receptive ovules. Yet another possibility is insect damage to the cones. I observed that some cones were curved, which is a sign of insect damage (Don Piggot, personal communication).

Low seed-set was a problem for the project design, causing an initial knock-on effect. I wanted to evenly represent the different categories of parent material (constitutive resin canal crosses, traumatic resin canal crosses, sclereid cell crosses, control crosses). This was not possible. Not only was I forced to choose crosses that appeared to have healthy cones, but I was further limited by the low number of filled seeds per cone. The average ranged from 1 to 25 seeds per cone for the controlled-crosses. A squirrel would have quit. As a result, I could not evenly represent the

different crosses for the resistance trait categories. This brought about an imbalance in the experimental design from the start.

Low seed set is also a practical concern for producing SE cultures. Producing SE cultures is labour-intensive. To avoid contamination it is important to not damage the seeds by breaking the seed coat. Each scale must be individually removed from the cone. If seed-set is low, every filled seed becomes valuable. When a cone contains few filled seed, it takes much greater effort.

Using material from the Sitka spruce weevil-resistance breeding program to produce SE clonal lines is like being at the bottom of a tree-breeding filter. The program began with a broad screening program for resistance that included provenances from much of the range of Sitka spruce (King and Alfaro 2009). This led to a more focused screening program. This program used seeds collected along a transect that passed through an original resistant provenance. Trees from this program were characterized for possible mechanisms of resistance. These mechanisms were then identified in specific genotypes (King and Alfaro 2009). Now by creating clonal lines, the potential exists to look at how variable these traits are within genotypes and how consistently they develop.

**Induction**

Induction is the first step in the process of somatic embryogenesis. Zygotic embryo or megagametophyte explants are placed onto induction medium containing two plant growth regulators, 2,4 – dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BA) (Stasolla and Yeung 2003). In some cases, exposing zygotic explants to these conditions triggers the proliferation of immature somatic embryos. These immature

embryos are composed of an embryonic mass of densely cytoplasmic cells, and attached suspensors of elongated, vacuolate cells (Stasolla and Yeung 2003).

The mean induction rate was 7 % in this project. This rate is low compared to induction rates published for spruce. Park (2002) suggests that average induction rates greater than 65 % are possible for spruce species (*Picea glauca*, *P. mariana* and *P. abies*) when immature embryo explants are used. The only published initiation rate for Sitka spruce is from Krogstrup et al. (1988), which was also the first paper on Sitka spruce somatic embryogenesis. The initiation rate given for this study was 3-5 %. Other studies have used Sitka spruce somatic embryo lines (Gale et al. 2008; Find et al. 1998; Selby et al. 1996) but initiation rates were not given. Since Krogstrup et al. (1988) published this initiation rate, great effort has been made in developing more efficient methods of induction (Park 2002).

High induction rates are important for practical applications of somatic embryogenesis. For tree breeders to use SE lines for clonal propagation, they must be able to induce enough genotypes to maintain desired levels of genetic diversity (Park 2002). Induction rates must also be high enough to make induction from elite crosses feasible (Park et al. 1994). Also, an important practical consideration is the labour-intensive nature of SE: this is a costly process. Zygotic embryo explants are dissected individually from seeds and placed onto induction medium. The lower the induction rate, the greater the wasted effort.

Low induction rates restricted our study design. Low induction rates resulted in the greatest loss of material during the entire process, from breeding to seedling establishment. We wanted to evenly represent the three groups of controlled-pollinations

(sclereid cells, traumatic resin canals, constitutive resin canals), the control group and the open-pollinated group. The mean induction rates for these groups were not even. The constitutive resin canal group had the highest rate of induction, the open-pollinated the lowest, with the others ranging in between. Having variable rates of induction, ranging from a low of 2 % to a high of only 15 %, meant that our study design was unbalanced. The induction phase limits each following stage of the SE process because without induced cultures, there is no material for the following stages. However, finding a wide range in induction rates between groups given identical induction treatments is not uncommon. Similar results were found by Park et al. (1993) for white spruce families. They found the mean induction rate for families ranged from 3.3 % to 54.6 %, with an average of 30.5 %.

Within the groups, the induction rates varied between individual crosses. For example, within the traumatic resin canal group, explants from only three of seven crosses were inducible. These three crosses were, themselves, not represented evenly. One cross had seven genotypes that yielded established cultures, while the other two crosses each had one genotype that became established. These results suggest it is necessary to start with much larger numbers of explants from a larger number of crosses. This would best ensure an adequate representation of desired crosses.

Since induction rates are critical to successful SE, an effort has been made to understand the factors affecting induction. I am aware of only one study that shows the stage-by-stage losses during the entire SE process: this was done on white spruce by Yill-Sung Park and co-workers in 1993 and 1994. The factor with the single greatest influence on induction is genotype. Induction of somatic embryogenic tissue is under

strong additive genetic control (Park et al. 1993). This means that some genotypes have the inherent ability to produce somatic embryos, and others do not. The genetic basis is not known, but it is likely related to control of cleavage polyembryony (von Aderkas et al. 1991). Genetic control likely explains much of the variation in induction rates in our study. It means that induction success is unpredictable. Some genotypes will be recalcitrant; there is no way around that. The best solution for a study like ours is to increase the number of crosses and explants in hopes of finding enough SE-capable genotypes.

According to Park et al. (1993), the developmental stage of the zygotic embryo explant affects induction. They found that immature zygotic embryo explants produced more embryogenic cultures than mature zygotic embryos. In this study, I collected cones from July to August 2008. Over this time, the zygotic embryos developed from immature embryos too small to be dissected from the megagametophyte into mature embryos with cotyledons. It is possible that some of the variation seen in induction rates between crosses may have resulted from the different developmental stages of the zygotic explants.

Park et al. (1993) also suggest that storing cones at 4 °C before extracting zygotic embryos affects induction rates. Cold-stored cones had lower induction rates than cones used immediately after harvest. I also stored some cones at 5 ± 1 °C for up to three weeks before the seeds were extracted. This may explain some of the variation in induction rates. However, storing cones is a necessary evil if there is distance between the seed orchard and the lab, not to mention if there is a restricted labour pool doing the dissecting.

It is also possible that the type of medium or the plant growth regulators chosen affected the induction rate. I used modified Litvay's medium as did von Aderkas et al. (2007) for interior spruce (*Picea glauca x P. engelmannii*). A preliminary trial using this media with Sitka spruce confirmed it was effective in inducing somatic embryogenesis. However, other types of media can induce somatic embryogenesis in spruce. Krogstrup et al. (1988) used variations of Murashige and Skoog media for induction of Sitka spruce. This medium has a very different nutrient composition from Litvay's medium. Krogstrup et al. (1988) also included kinetin as a plant growth regulator, in addition to 2,4-D and BA. Park (2002) suggests that modifying media may help to optimize induction rates.

Genotypic differences likely explain the variation in morphological responses observed for the induction treatment (Park et al. 1993). Some genotypes produced embryogenic tissue, whereas others consistently produced green or white callus. Once embryogenic cultures became established, they showed differences in tissue texture, colour and growth rate. It is not clear whether these morphological differences are heritable: a deliberate breeding experiment would be required to verify this. Implied support lies in the Park et al. (1993) study in which they showed that SE was heritable in white spruce.

**Maturation**

Maturation is the most critical step in the SE process. Somatic embryos transition from immature embryos into histologically differentiated mature embryos within a period of 6-8 weeks (Stasolla and Yeung 2003). They begin as aggregations of embryonal masses with their suspensors, developing into mature embryos with root and shoot meristems, embryonic stele and cotyledons. This transition is triggered by a change in

medium, including a change in plant growth regulators (PGRs). For induction and maintenance, 2,4-D and BA are used to promote tissue proliferation. Removing auxin and cytokinin stops cleavage of immature embryos. Replacing them with abscisic acid leads to the formation of numerous, high quality mature somatic embryos (Stasolla and Yeung 2003). Attaining a high and consistent maturation rate is key in successfully completing the SE process. Mature embryos are distinguished not only by their morphological status, but by their acquisition of physiological traits such as reserves of starch, fat and protein (Stasolla and Yeung 2003).

Most genotypes (88.1 %) placed on maturation medium produced at least one mature somatic embryo. This rate is higher than the most recently published rate for Norway spruce, where 67.1 % of genotypes produced mature embryos (from table in Wang et al. 2009). When grouped by family, Wang et al. (2009) report a mean maturation rate of 64 %, ranging from 17 % to 87 %. However, Wang et al. (2009) do not define the number of embryos they required to consider a culture capable of maturing. This is not a standardized aspect. I included any culture that could produce at least one mature embryo on any maturation plate.

The variation in the number of embryos produced by different genotypes necessitated a system of categorization, which may appear arbitrary but had a practical context. My goal was to select genotypes to germinate and grow into trees. Initially, I wanted to produce even numbers of trees from the three categories of resistance trait crosses. To do this, I needed to select genotypes that had produced sufficient numbers of embryos. I intended to have at least 30 embryos of each genotype to germinate, and in turn have a minimum of twelve trees per genotype. Genotypes producing greater than 30

embryos per plate were ideal, but multiple plates producing between 10 and 30 embryos were also adequate. About three-fifths of all genotypes were both consistent and prolific enough to be considered useful. The other two-fifths either produced too few mature embryos or were too inconsistent.

It was apparent in this study that genotypes varied in their capacity to produce mature somatic embryos. Just as the ability to induce somatic embryogenesis is under additive genetic control, the ability to produce mature somatic embryos is determined in part by genetic factors (Wang et al. 2009; Park et al. 1994). Park et al. (1994) found that 7.8 % of the genetic variance for maturation of somatic embryos was explained by general combining ability. This explains why some genotypes are recalcitrant to maturation (Wang et al. 2009). They do not have the genetic ability to produce mature embryos.

Genotype-specific characteristics in embryo appearance were observed. Good quality embryos from different genotypes not only looked different, but had sizes and shapes that were clearly distinct. On the negative side, some genotypes were vitrified with deformed embryos and others produced precocious shoots. These extreme morphological responses were attributable to genotype (Park et al. 1994).

It is also clear from my study that there is variation in the number of mature embryos produced by a given genotype. Researchers have looked to the interaction between ABA and somatic embryos to understand the control of maturation capacity. Abscisic acid is the plant growth regulator applied to trigger maturation (Gutmann et al. 1996). Kong and von Aderkas (2007) compared the ability of genotypes varying in maturation capacity in their uptake of exogenous abscisic acid. They found that

genotypes with a high capacity to produce mature somatic embryos utilized a greater amount of exogenous abscisic acid than those with a poor maturation capacity.

More recently, Robinson et al. (2009) have applied a metabolomics approach to the question of why some genotypes produce more mature embryos than others in loblolly pine. They wanted to determine if there was a particular metabolic profile present in immature somatic embryogenic cultures that could predict maturation capability. Their results suggested that there was a strong transition in metabolic activity as immature embryos developed on maintenance medium. Although this may appear to be obvious, they suggest that the more developmentally advanced the immature embryos were when placed on maturation medium, the more successful they would be in forming mature embryos. Their results also suggested that stress responses may affect successful maturation. They found that some stress-related metabolites (serine, proline, arabitol) correlated negatively with the ability to produced mature embryos. However, in complete contradiction to this result, they also found that another stress-related metabolite, sorbitol, was associated with successful mature embryo production.

In an SE system the cumulative success at a given stage depends on the success of all previous stages. It may be possible that some genotypes that produced few embryos or poor quality embryos could produce more or better quality embryos if alterations were made to medium hormone supplements and conditions used (Park 2002). In a project of a larger scale, genotypes that are from very desirable crosses may be tested on a variety of media in hopes of producing more or better quality embryos. In a small-scale project like this, this is not possible due to the effort required. However, this could provide one way to attain somatic embryos from more desired crosses.

**Germination**

Germination in somatic embryos differs from germination in zygotic seeds. Somatic embryos are not in a seed. They rely on medium free from plant growth regulators to begin germination (Park et al. 1998). The sequence of events in somatic embryo germination is also different from zygotic seeds, as described by Pullman et al. (2003) for loblolly pine. In somatic embryos, first the embryo becomes green and the hypocotyl elongates. Then the cotyledons expand. Lastly, the root begins to elongate. In zygotic seeds, the root emerges first, followed by shoot growth (Pullman et al. 2003).

There is no consensus in the literature on how to evaluate germination in somatic embryos. Some authors record germination as root elongation (Roberts et al. 1991). Others require root, shoot and hypocotyl elongation (Attree et al. 1990). I used hypocotyl and root elongation together as a measure of germination. Hypocotyls and roots in conifers are, to a degree, continuous (von Guttenberg 1961). Hypocotyl and root elongation together contribute to root emergence in both zygotic and somatic embryos (Pullman et al. 2003). Elongated hypocotyls and roots are easily and clearly observed in embryos placed on to germination medium.

Rates of germination for large numbers of genotypes are difficult to find in the literature. The focus of most studies is on improving germination by comparing different treatments (Roberts et al. 1990; Roberts et al. 1991). These studies typically use only a very small number of genotypes (Roberts et al. 1990; Roberts et al. 1991; Kvaalen and Appelgren 1999). This makes it unreasonable to draw comparisons with this study where I used 45 different genotypes. The most recent and relevant comment on germination comes from Park (2002). Here, Park (2002) suggests that about 80 % of spruce genotypes that can be induced to produce embryogenic cultures are also able to produce

plants. From this, I assume that germination rates for embryogenic genotypes of spruce must be greater than 80 %. Therefore, that 44 of 45 genotypes germinated seems to be within reason.

Germination rates of up to 90 % have been reported for spruce (Roberts et al. 1990). This number refers to the *percent of embryos* that successfully germinate, as opposed to the number of genotypes that can germinate. The mean germination rate of 80 % in this study is slightly lower. As previously mentioned, Roberts et al. (1990) used a small number of genotypes for their estimation. This rate may not be comparable, especially if only genotypes producing the highest quality embryos were used for germination in their study.

The percentage of embryos that germinated *per genotype* varied. In a third of genotypes, every embryo germinated. These genotypes should be considered the most valuable simply because they are reliable. Another 19 of 45 genotypes had germination rates greater than 60 %. Before beginning the method development, I had initially estimated the number of embryos required to produce twelve trees based on this germination rate. Of the remaining 12 genotypes, 10 had germination rates greater than 40 %. Although less reliable, these genotypes should be considered useful if they were also prolific producers of mature embryos. Only one genotype did not germinate at all. This represents a dead-end in the SE process. It took eight months from induction to reach the germination phase. Genotypes that pass successfully through induction and maturation, but fail to germinate are a great waste of time and effort.

If Sitka spruce is like white spruce, the ability of genotypes to germinate is, like the other stages of somatic embryogenesis, under additive genetic control (Park et al.

1994). In white spruce, approximately 1.5 % of variance in germination was described by the general combining ability (Park et al. 1994). This was lower than for the other stages of somatic embryogenesis. Genetic control explains the differences between genotypes in their ability to germinate.

It was apparent that germinants varied in quality. Good quality germinants developed shoots with primary leaves, had a straight hypocotyl, and developed a root. Poor quality germinants were deformed and some were vitrified. Similar variation in germinant quality was mentioned by Park et al. (1994) for white spruce and by Roberts et al. (1991) for Sitka spruce. Partial desiccation with a high relative humidity treatment has been suggested as a method to improve germinant quality in Sitka spruce (Roberts et al. 1991) and white spruce (Roberts et al. 1990). I did not apply a partial desiccation treatment to the germinants in this study as there are no reliable methods available using calibrated technology (Percy et al. 2001).

Another factor that may influence germination rates is the degree of maturity of the embryos used. Once embryos have reached morphological maturity, they still need to undergo physiological maturation (Stasolla et al. 2003). This is true of all embryos, not just somatic (Edwards 1980). Perhaps there is variation in the rate of maturation of somatic embryos. It could also be that some genotypes produce a lot of embryos that do not reach full maturity and therefore cannot germinate.

**Growth**

Acclimatization proved to be the single most difficult step throughout all stages of this project. Only about 5.5 % of germinants survived. Conversion rates of 90 % have been reported for high quality Sitka spruce germinants (Roberts et al. 1991). In this

project, there were two obvious points of loss. First, many germinants died because of difficulties in controlling the humidity of the fogging chamber. This, in turn, created conditions perfect for a fungus gnat infestation to occur. These two factors – one abiotic, the other biotic - likely explain the low conversion rate.

The method used to acclimatize the germinants in this study was troublesome. Compared to other methods, however, it appears relatively conservative. Some studies report transferring germinants directly to moistened solid growing media (Grossnickle et al. 1994). Others suggest covering the germinants with a layer of plastic wrap to retain humidity (Roberts et al. 1990; Webster et al. 1990). Germinants are acclimatized in a growth chamber, before being placed into a greenhouse (Grossnickle et al. 1994). Some researchers use a controlled misting system to gradually decrease the humidity (Khlifi and Tremblay 1995; Lamhamedi et al. 2000). The simplest albeit most expensive method is to put germinants in a temperature and humidity controlled greenhouse with an automatic fogging system. CellFor, a commercial SE company, uses such facilities (personal observation), as did Hogberg et al. (2003).

I would not assume that because some (5.5 %) of the germinants were successfully acclimatized that all would acclimatize. Ability to acclimatize is thought to be genotype-dependent, like all other stages of the SE process (Stasolla et al. 2003). The quality of the germinants also affects their ability to acclimatize. Roberts et al. (1991) showed that for Sitka spruce, only 39.3 % of low quality germinants could be successfully acclimatized, compared to 90 % for high quality genotypes. Poor quality germinants included those appearing vitrified. Some of the Sitka spruce germinants in this project appeared vitrified to some degree.

The germinants that were successfully converted to plants were from a number of genotypes and from a variety of crosses. This shows that it is possible to take material from controlled-crosses through the SE process, and end up with a good representation of these crosses at the end. Although the method I used for acclimatization was problematic, I still showed that it is possible to produce cloned trees. To improve conversion, a complex expertise in acclimatization, i.e. vapour pressure deficit effects on seedling physiology, is required.

**Cryopreservation**

Cryopreservation provides a great advantage to somatic embryogenesis as a method of clonal propagation. Embryogenic cultures can be stored in liquid nitrogen, allowing time to grow and to test the resulting clonal plants. The cryopreserved cultures can later be thawed, bulked-up and used in mass propagation (Park et al. 1998). Cryopreservation also allows researchers to re-visit favourite genotypes repeatedly, with reduced concern over the loss of embryogenicity in cultures over time (Klimaszewska et al. 1992 and references therein). Cryopreservation has been achieved for Sitka spruce using other methods (Find et al. 1993; Find et al. 1998).

Typically, cryopreservation methods involve a series of steps that reduce damage to cells during freezing and thawing. Cultures are treated with sorbitol and dimethyl sulfoxide (DMSO) - an osmoticant and a cryoprotectant, respectively - to reduce damage caused by intracellular ice formation. Cultures in solution are slowly frozen using a programmable freezer to a temperature of -40 °C, and then immersed in liquid nitrogen (-196 °C) (Percy et al. 2001; Kartha et al. 1988; Klimaszewska et al. 1992; Cyr et al. 1994). DMSO is often used as a cryoprotectant (Kartha et al. 1988; Klimaszewska et al.

1992; Cyr et al. 1994). However, DMSO has been found to alter genetic stability in conifers (Aronen et al. 1999). Alternative cryoprotectants have been used: Touchell et al. (2002) used sorbitol and a plant vitrification solution.

Pretreatments free of cryoprotectants do not seem to appear in the literature, at least in regards to cryopreservation. Temperature pretreatments have been shown to affect desiccation tolerance in white spruce (Pond et al. 2002). They showed that an eight-week temperature treatment of 5 °C improved the ability of mature somatic embryos to tolerate desiccation. As well, von Aderkas et al. (2007) showed that temperature pretreatments can affect the cold tolerance of somatic embryos. However, I am not aware of a study that presents a method of cryopreservation using a temperature pretreatment. I was able to show that Sitka spruce embryogenic cultures can be cryopreserved using a temperature pretreatment, in which cultures are placed on semi-solid modified maturation medium and kept at 5 °C for 8-12 weeks, prior to direct immersion into liquid nitrogen. I found that 31 % of Sitka spruce genotypes could produce embryogenic cultures after being cryopreserved with this method. This demonstrates that Sitka spruce can be successfully cryopreserved without the use of the potentially damaging cryoprotectant DMSO. Further, this method is simple and does not require an expensive programmable-freezer. More work is needed to optimize this method for Sitka spruce.

Genotypes showed a variable response to the pretreatment: some genotypes grew and matured, while others showed little growth during the pretreatment. In general, it seems that genotypes showing growth and maturation during the pretreatment were more likely to survive cryopreservation. The genotypes that showed little or no growth during

the pretreatment were less likely to survive cryopreservation. Norgaard et al. (1993) compared morphological and physiological traits of cultures with their ability to survive cryopreservation. They found that survival correlated neither with culture morphology, nor with maturation capability. They did, however, suggest a weak correlation with growth rate. However, this was not in response to a pretreatment. To understand whether response to pretreatment relates to survival after cryopreservation, a quantitative study would be required.

**Development**

The somatic embryos produced in this project developed normally (at least those genotypes considered to be of good quality). Mature embryos had distinct cotyledons, hypocotyls, meristems and root caps. This is important, as the goal of SE systems is to generate embryos with these given features, which, in turn, can be germinated and grown into trees (von Aderkas et al. 2002). Abnormalities, such as misshapen form or extremely vacuolated, expanded cells (Gutmann et al. 1996) or large intercellular airspaces (Kong and Yeung 1994) were not visible in the Sitka spruce somatic embryos.

Idioblast cells were visible in the cotyledons and the hypocotyls of the mature embryos. These cells have been reported previously in hybrid larch somatic embryos (Gutmann et al. 1996; von Aderkas et al. 2002). Von Aderkas et al. (2002) described idioblasts in hybrid larch somatic embryos as large cells rich in lipids and starch, that often contain large numbers of vacuoles. Idioblasts are also found in zygotic embryos of conifers. Spurr (1950) described idioblasts in zygotic embryos of pine. Their origin has been described as schizogenous and lysigenous (Cutler et al. 2008).

A number of possible functions for embryonic idioblasts have been suggested. Hutchison (1917) described them as mucilage cells, while Schopf (1943) described them as secretory cells. Spurr (1950), also referred to them as secretory cells, and suggested they are related to the development of the resin canal system in conifers. Von Aderkas et al. (2002) suggest that the function of idioblasts in conifer somatic embryos is yet to be understood.

**Conclusions**

Somatic embryogenesis can be used to create a robust system for studying phenotypic variation in conifers. Here, I have shown that it is possible to select material from a breeding program, take this material through the SE process (induction, maturation, germination, acclimatization), and produce fully functional seedlings at the end. It may seem that setting up an SE system is more work than it is worth in terms of time, effort and therefore, money. This is not true. Investigative forestry projects commonly take decades. The Sitka spruce weevil-resistance program has been running for almost 30 years (King and Alfaro 2009). Trees are large and complex. They take a lot of effort to understand. With this in mind, taking two years to set up a cloning system that can help dissect a large problem, such as weevil-resistance traits, is not at all unreasonable. The trees that come from this project can be planted and studied for years to come. Embryogenic lines were also successfully cryopreserved. This means that if an interesting genotype-specific trait is found, we have the potential to re-propagate that exact genotype from our cryopreserved stores for further study. Traits can then be traced from the very earliest stages of embryogenesis. Genotypes can be studied from all perspectives: morphological, anatomical, genetic and biochemical.

Genotypic and phenotypic variation seeped into every stage of this project. The effects of genotypic variation may hinder progress through the stages of SE. Clearly not all genotypes are amenable to propagation by somatic embryogenesis. Looking from a wider perspective, phenotypic variation within and between genotypes is evident from the early stages of embryogenesis and occurred throughout this project. Given the relatively controlled and consistent environment provided for these tissue cultures, this variation is

proof of sensitivity and responsiveness of genotypes to environmental conditions during embryogenesis.

# Chapter 3: Perspectives

A cloning system for pedigreed trees has many potential uses. Here, the focus is on resistance traits to the white pine weevil in Sitka spruce. This system can provide clones that are grown from completely independent embryos. This allows us to observe whether a given trait will develop independently in genetically identical, but separate organisms. Once grown into trees, somatic ramets can provide replicates. This increases statistical confidence when genotypes are phenotypically characterized. Independently developed clones can provide an understanding of how consistently these bark traits arise.

Literature on the development of constitutive resin canals is scarce. It is surprising that so little information is available on the resin canal system given the importance of resin canals as a defensive mechanism in conifers. The most detailed account, by Spurr (1950), suggests idioblast cells in pine embryos as being related to constitutive resin canals. Spurr (1950) does not include development of the resin canal system between germination and seedling growth. The question then remains, are idioblast cells related to the development of constitutive resin canals? This is the type of question for which an SE system is best suited.

In this study, some embryogenic lines were derived from parent genotypes with extreme phenotypes for constitutive resin canals. This provides the ideal system for answering this question of how resin canals develop. Somatic embryos from these clonal lines could be sectioned and the idioblast cells could be counted. Results from this survey could be compared to information about the parent genotypes to see if a

correlation exists. Embryos could be grown into seedlings. Growing seedlings could be sampled and sectioned to monitor the development of the resin canal system. This could provide a complete understanding of the development of constitutive resin canals.

Understanding the development of traits is of basic scientific interest. Mature trees are complex. How *does* that happen? Understanding the development of traits is also practical. Recent studies have shown that some stages of spruce embryogenesis influence the formation of adult phenotypes. Kvaalen and Johnsen (2008) showed that bud set in mature trees is affected by temperature during embryogenesis, and the effects are permanent. The next question is whether embryological characteristics related to other adaptive traits may be detected in embryos. If it was established that specific traits in mature trees could be related to specific embryological traits, then seeds or somatic cultures could be screened. This could have great implications for clonal forestry. For example, if the abundance of idioblast cells in embryos were related to abundance of constitutive resin canals in mature trees, somatic clonal lines could be screened for resistance traits. It would be unnecessary to wait for a sample tree to grow to see if these traits would develop. This could eliminate years from breeding cycles.

I have shown that it is possible to create a SE system designed to tackle a specific set of phenotypic traits. Here, it was resistance traits to the white pine weevil in Sitka spruce. This type of system could be developed for any number of adaptive traits in any conifer species amenable to somatic embryogenesis. Systems could allow the methodical dissection of phenotypic variation during any stage of development. There was a range of genotypic variation and phenotypic plasticity evident throughout this project. How this variability will relate to the phenotypes of the embryogenic lines as they develop

remains to be seen. Studying the phenotypic variability, both between and within genotypes, will lead to a better understanding of the consistency of these weevil resistance traits in Sitka spruce.

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**Appendix 1. Proportion of germination by genotype.**

**Appendix 1**

| Sclereid Cells | Cross | Genotype | Number of Embryos Germinated | Total Number of Embryos | Arcsine Transformed Proportion of Germination |
|---|---|---|---|---|---|
| **High female, high male** | 1020x1153 | BC1 | 25 | 48 | **0.81** |
| | 946x1020 | AB6 | 20 | 48 | **0.70** |
| | | AB2 | 34 | 48 | **1.00** |
| | | AB8 | 48 | 48 | **1.50** |
| | 1242x1020 | DB8 | 36 | 48 | **1.05** |
| | | DB9 | 40 | 48 | **1.15** |
| | | DB1 | 48 | 48 | **1.50** |
| | | DB6 | 48 | 48 | **1.50** |
| **High female, low male** | 1242x1018 | DF9 | 10 | 23 | **0.72** |
| | | DF8 | 17 | 19 | **1.24** |
| **Low female, high male** | 1018x1020 | FB5 | 25 | 48 | **0.81** |
| | | FB1 | 34 | 48 | **1.00** |
| **Low female, low male** | 1018x1253 | FG3 | 7 | 15 | **0.75** |
| | | FG5 | 35 | 48 | **1.02** |
| | 1253x1018 | GF2 | 21 | 48 | **0.72** |
| | | GF4 | 21 | 48 | **0.72** |
| | 860x1018 | EF6 | 17 | 48 | **0.64** |
| | | EF1 | 17 | 32 | **0.82** |
| | | EF5 | 30 | 48 | **0.91** |
| | | | | **Mean Arcsine Value** | **0.98** |
| | | | | **Back transformed mean proportion** | **0.69** |
| | | | | **Range** | **0.35-1.00** |

**Appendix 1 continued . . .**

| Constitutive Resin Canals | Cross | Genotype | Number of Embryos Germinated | Total Number of Embryos | Arcsine Transformed Proportion of Germination |
|---|---|---|---|---|---|
| **High female, high male** | 1043x1018 | MF4 | 33 | 48 | **0.98** |
| | 1209x1018 | NF2 | 36 | 48 | **1.05** |
| | | NF3 | 41 | 48 | **1.18** |
| | | NF1 | 48 | 48 | **1.50** |
| | 1043x1209 | MN5 | 48 | 48 | **1.50** |
| | | MN11 | 48 | 48 | **1.50** |
| | | MN15 | 48 | 48 | **1.50** |
| **Low female, low male** | 1241x1010 | OI3 | 42 | 48 | **1.21** |
| | | OI9 | 48 | 48 | **1.50** |
| | | OI2 | 48 | 48 | **1.50** |
| | 1241x945 | OL7 | 36 | 48 | **1.05** |
| | | OL13 | 42 | 48 | **1.21** |
| | | OL6 | 47 | 48 | **1.43** |
| | 1010x945 | IL11 | 48 | 48 | **1.50** |
| | | IL19 | 48 | 48 | **1.50** |
| | | IL32 | 48 | 48 | **1.50** |
| | | | | **Mean Arcsine Value** | **1.35** |
| | | | | **Back transformed mean proportion** | **0.95** |
| | | | | **Range** | **0.67-1.00** |

**Appendix 1 continued . . .**

| Controls | Cross | Genotype | Number of Embryos Germinated | Total Number of Embryos | Arcsine Transformed Proportion of Germination |
|---|---|---|---|---|---|
| | 437x444 | SR4 | 22 | 48 | **0.74** |
| | | SR2 | 31 | 48 | **0.93** |
| | | SR5 | 33 | 48 | **0.98** |
| | 215x444 | TR13 | 0 | 16 | **0.13** |
| | | TR11 | 23 | 48 | **0.77** |
| | | | | **Mean Arcsine Value** | **0.70** |
| | | | | **Back transformed mean proportion** | **0.41** |
| | | | | **Range** | **0.00-0.69** |
| **Open-pollinated** | | | | | |
| | 1020 | B1 | 30 | 48 | **0.91** |
| | | B2 | 35 | 48 | **1.02** |
| | 1010 | I2 | 40 | 48 | **1.15** |
| | | I4 | 48 | 48 | **1.50** |
| | | I6 | 48 | 48 | **1.50** |
| | | | | **Mean Arcsine Value** | **1.22** |
| | | | | **Back transformed mean proportion** | **0.88** |
| | | | | **Range** | **0.63-1.00** |
| | | | | **Mean of all arcsine values** | **1.11** |
| | | | | **Back transformed mean of all arcsine values** | **0.80** |
| | | | | **Range** | **0.00-1.00** |

**Appendix 2. Number of seedlings established per genotype.**

**Appendix 2**

| Sclereid Cells | Cross | Genotype | Number of Germinants Planted | Number of Established Seedlings |
|---|---|---|---|---|
| **High female, high male** | 1020x1153 | BC1 | 20 | 0 |
| | 946x1020 | AB8 | 20 | 0 |
| | | AB6 | 20 | 1 |
| | | AB2 | 20 | 2 |
| | 1242x1020 | DB6 | 20 | 0 |
| | | DB9 | 20 | 2 |
| | | DB8 | 20 | 3 |
| | | DB1 | 20 | 4 |
| **High female, low male** | 1242x1018 | DF8 | 9 | 0 |
| | | DF9 | 13 | 1 |
| **Low female, high male** | 1018x1020 | FB5 | 20 | 0 |
| | | FB1 | 20 | 2 |
| **Low female, low male** | 1018x1253 | FG3 | 9 | 3 |
| | | FG5 | 20 | 3 |
| | 1253x1018 | GF4 | 20 | 0 |
| | | GF2 | 20 | 1 |
| | 860x1018 | EF5 | 20 | 0 |
| | | EF6 | 20 | 1 |
| | | EF1 | 17 | 1 |

**Appendix 2 continued . . .**

| Constitutive Resin Canals | Cross | Genotype | Number of Germinants Planted | Number of Established Seedlings |
|---|---|---|---|---|
| **High female, high male** | 1043x1018 | MF4 | 20 | 0 |
| | 1209x1018 | NF2 | 20 | 0 |
| | | NF3 | 20 | 2 |
| | | NF1 | 20 | 2 |
| | 1043x1209 | MN5 | 20 | 0 |
| | | MN11 | 20 | 0 |
| | | MN15 | 20 | 0 |
| **Low female, low male** | 1241x1010 | OI9 | 20 | 0 |
| | | OI2 | 20 | 0 |
| | | OI3 | 20 | 2 |
| | 1241x945 | OL13 | 20 | 0 |
| | | OL7 | 20 | 1 |
| | | OL6 | 20 | 1 |
| | 1010x945 | IL19 | 20 | 0 |
| | | IL32 | 20 | 0 |
| | | IL11 | 20 | 2 |

**Appendix 2 continued . . .**

| Controls | Cross | Genotype | Number of Germinants Planted | Number of Established Seedlings |
|---|---|---|---|---|
| | 437x444 | SR5 | 20 | 0 |
| | | SR4 | 19 | 3 |
| | | SR2 | 20 | 4 |
| | 215x444 | TR13 | 7 | 0 |
| | | TR11 | 20 | 2 |
| **Open-pollinated** | | | | |
| | 1020 | B2 | 20 | 0 |
| | | B1 | 20 | 2 |
| | 1010 | I2 | 20 | 0 |
| | | I4 | 20 | 0 |
| | | I6 | 20 | 2 |
| | | **Total** | **854** | **47** |
| | | **Overall Conversion Rate (%)** | **5.5** | |