The Technology of Clonal Forestry of Conifers

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Clonal forestry is the planting of vegetatively propagated ramets, whose clonal identity is maintained, from known and tested ortets for the commercial production of trees. This occurs regularly in poplar and other broadleaf cultivation but is rare in conifer cultivation. More common in conifer cultivation is vegetative multiplication which is the vegetative propagation of mixtures of genotypes of known and tested genetic crosses where the identity of individual clones is not maintained. In this paper, clonal forestry of conifer species only will be discussed.

One of the major problems facing the implementation of clonal forestry is phase change. Wareing has defined phase change as the series of changes that occur when a tree passes from the juvenile phase in which there is the ability to initiate adventitious structures and a general absence of flowering to the mature phase where flowering is common and the ability to initiate adventitious structures is lost or dramatically reduced. These changes are epigenetic and have large effects on the physiology of the tree. Greenwood has suggested that maturation is the process of phase change that results in relatively permanent developmental changes. This might mean that phase change reversal might not be possible in somatic cells i.e. rejuvenation might not be possible.

The process of phase change is continuous but it can be split up into six broad phases.

1. The pre-embryonic phase, which starts immediately following sexual fusion until full formation of the zygotic embryo. Development is most plastic and most in vitro techniques can be applied.

2. The embryonic phase which is for the duration of the mature zygotic embryo. The development is still very plastic and most in vitro techniques can be applied. The ability to form embryogenic tissue is decreasing.

3. The seedling phase following the germination of the seed. The plants tend to be in free growth, juvenile foliage is produced and sexual reproductive structures are rare. Tissue development is still fairly plastic and adventitious structures are relatively easy to initiate but embryogenic tissue is very difficult to induce.

4. The transition phase where growth characteristics such as branch habit, foliar morphology growth rate and wood production gradually change. The rooting of cuttings and the ability to tissue culture decline during this period.

5. The mature phase where sexual reproduction is maximised and morphological characters are stable. The rooting of cuttings and tissue culture are difficult if not impossible.

6. The degenerative or senescent phase where the order within the tree breaks down and results in death of the tree.

The process of phase change presents many problems for clonal forestry. When the tissue is most plastic and amenable to vegetative propagation, the genotype is untested and a prediction of final performance is improbable. When the genotype is tested and a prediction of final performance possible, the tissues are much less plastic and vegetative propagation is difficult or impossible. There are two possible ways in which this problem may be circumvented. The mature tissues could be rejuvenated to the juvenile form allowing vegetative propagation to be used. Alternatively, the tissues could be maintained in the juvenile form in some way whilst the genotype was tested. Good genotypes could then be vegetatively propagated from the juvenile form of the genotype.

There are two possible types of rejuvenation that could occur in conifers i.e. zygotic and somatic. Zygotic rejuvenation occurs naturally in trees and happens when mature tissues undergo a series of genetic changes during meiosis that result in the reversal of the epigenetic changes that occur during maturation. The zygote that is formed following gamete fusion demonstrates juvenile characters. Somatic rejuvenation does not occur naturally in trees and perhaps is a theoretical rather than a practical proposition. To achieve somatic rejuvenation, it would be necessary to reverse the epigenetic changes that occurred during maturation by repeated application of a stimulus to the mature tissue. To ensure that somatic rejuvenation had occurred, the tissue would need to be matured to ensure that the tissue underwent all the normal stages of maturation in the correct sequence over the normal time scale.

Should complete rejuvenation of the mature genotypes prove not to be possible, other methods must be found that can keep the genotype in the juvenile state whilst it is being tested. Hedging has not proved to be very successful and repeated micropropagation is far too expensive. The best option is cryopreservation where the tissue would be stored at ultra low temperatures, usually –196c where the tissue goes into stasis and undergoes no change. Juvenile tissues from the ortet would be put into cryopreservation whilst the ortet was tested. Ramets of superior tested ortets would then be generated from the cryopreserved tissue and used in clonal forestry.

A number of propagation techniques are available for use in clonal forestry and they tend to work in different parts of the phase change process. Stem grafting works in

all phases. Cuttings succeed best in the juvenile and early adolescent phases. Micropropagation works best with juvenile tissues whereas somatic embryogenesis is successful only with mature or immature embryos at the moment.

There is a long history of the study and use of vegetative propagation techniques in Forest Research. In the 1950's'and 1960's, grafting and the rooting of cuttings were attempted with generally poor results. In the 1970's, intensive studies of the physiology of rooting and the grafting of Sitka spruce and both techniques were improved considerably. Tissue culture studies were started by funding a graduate study at Leicester University. In the 1980's, clonal multiplication was started commercially and intensive studies were made on tissue culture and rejuvenation. In the 1990's and to date, somatic embryogenesis, genetic transformation and cryopreservation were studied.

Grafting is the oldest of the vegetative propagation techniques. It was used by the Chinese as long ago as 1000BC and Aristotle wrote about it (384-322BC). It was widely used by the Romans and has been developed further over the millennia. Grafting has been used and developed in Forest Research over the last 50 years. Success rates with Sitka spruce (49%) are fair but acceptable. Those with Scots pine (72%) and Corsican pine (71%) are good and those with European larch (94%) and Japanese larch (97%) are excellent. Grafting has been used for the establishment of clone banks and seed orchards and for the rescue of valuable genetic material. It is unlikely that grafts would be used for the establishment of clonal forests of conifer species.

The use of cuttings does not have as long a history as grafting but *Cryptomeria japonica* has been rooted in Kyoto since about 1400 and there has been a written established procedure since 1697. The initial approach to cuttings propagation in Forest Research was based on a poor understanding of the physiology of coniferous species. In the 50's, 60's and early 70's attempts were made to root cuttings from trees that were too old and the results were very varied. A major project was initiated in the early 70's to propagate open pollinated plus tree progeny in progeny trials. The results were better than previously but still not acceptable. Research done elsewhere began to demonstrate that high levels of rooting could only be achieved if the donor stock plants were physiologically juvenile i.e. under three years old. The direction of the research then changed and a system for clonal multiplication, similar to that developed by Jochem Kleinschmitt in Germany, was developed. Improved seed was germinated and cuttings taken from the forced stock plants. The cuttings were rooted and a second cycle of cuttings was taken from the first and rooted. The system was commercialised and transferred to Delamere nursery in Cheshire, England. Clonal identity is not maintained in the system i.e. it is clonal multiplication as defined above. The Delamere nursery now produces about six million improved rooted cuttings per year for forest planting.
Cuttings are a powerful research tool and have been used for anatomical, physiological, genetic and pathological studies. They have been used in progeny

testing to enable the same genotype to be tested on a number of sights. They have been used for clonal multiplication but questions still remain if they will ever be used in clonal forestry as defined above. Cuttings can only be used in clonal forestry if there is a constant supply of juvenile but tested clonal. This might be provided by rejuvenation of mature tested genotypes but the evidence that complete rejuvenation to the juvenile genotype can occur is poor. Perhaps the only way that cuttings might be used in clonal forestry is that stock plants for cutting production might be generated from cryogenically stored and tested genotypes.

In Vitro or tissue culture techniques have been and are being developed in various conifer species. Tissue culture is the most recent of the vegetative propagation techniques to have been developed. The concept of totipotency was postulated in 1838 i.e. cells are autonomic and are capable of giving rise to new plants. However, it was many years before this concept was proved to be correct. It wasn't until the 1920's that organs were cultured. The auxins were characterised in the 1930's and angiosperm cells were finally cultured in the 1940's. It was the discovery of cytokinins in the 1950's that finally allowed media to be prepared with precision and tissue culture technology has developed rapidly since then. It wasn't until the 1980's that somatic embryogenesis in conifers was demonstrated but this technology has been extensively researched and is probably getting very near to commercial exploitation.

Callus cultures are relatively easy to induce in conifer species. The callus tends to grow well but it is very difficult to induce adventitious structures on its surface. Callus systems are not suitable for use as a vegetative propagation system for conifers.

Two possibilities exist for the micropropagation of spruces and larches *in vitro* using apical meristems i.e. adventitious and axillary budding. Adventitious budding is where tissues that are not destined to become apical meristems are induced to form meristems by the action of applied growth substances. Induction rates can be quite high but the subsequent media changes required for bud outgrowth, shoot elongation and rooting reduce the multiplication rate over time. Axillary budding is where the axillary buds formed in the apex of the juvenile *in vitro* plant are used to form the next generation of cultures. The system tends to use less media changes and multiplication rates per subculture cycle can be higher than in adventitious budding.

The system developed in Forest Research for the micropropagation of Sitka spruce is relatively simple. Seed is germinated in non-sterile conditions. The seedlings at the cotyledon stage are surface sterilised after the root system has been discarded. The sterilised explants are transferred to a hormone free medium and the cultures are maintained in a growth room under long days at a temperature of 20C. The apical meristem of the culture grows and during this growth the meristem produces axillary buds which can become axillary shoots. All of these different meristems become the cultures in the next cycle of cultures i.e. each culture cycle is both a multiplication

cycle and a bud development cycle. When required the micropropagated shoots are rooted under non-sterile conditions and weaned to form whole plants.

Shoot micropropagation has a number of uses. It can be used to produce large numbers of clonal plants for experimental purposes. It can be used to produce plants that have been manipulated *in vitro* or have been cryopreserved. At the moment, it is being used in Forest Research to produce rapidly large numbers of clonal rootstocks for cuttings propagation and the rooted plants will be used in a genetic gain trial.

It is possible that shoot micropropagation might be used in clonal forestry. As in a cuttings programme, micropropagation would require a supply of tested juvenile genotypes to be possible. These might be from rejuvenated or cryopreserved juvenile shoot tips. The clonal shoot cultures could subsequently be maintained under normal *in vitro* conditions as this apparently arrests the maturation process.

The *in vitro* technique that holds the greatest hope for clonal forestry is somatic embryogenesis, coupled with a few ancillary techniques. Haberlandt wrote in 1902 that "I am not making too bold a prediction if I point to the possibility that one could successfully cultivate artificial embryos from vegetative cells." Researchers had to wait until 1959 when the phenomenon was demonstrated in Daucas (carrot) and until the mid 1980's when it was demonstrated in a conifer species.

There are two types of embryogenesis that are possible in plants i.e. zygotic and somatic. Zygotic embryogenesis is the development of the embryo within the seed under natural biochemical and physiological control following the fusion of two gametes. Somatic embryogenesis is the development of embryo-like structures *in vitro* on tissues derived from somatic cells under artificial control.

In conifers, there are a number of tissues that could be used as the origin of embryogenic tissue but the ability to form this type of tissue or embryonyl suspensor mass (ESM) is generally restricted to the most juvenile stages in tree development. Formation of ESM is relatively easy in the pre-embryonic phase and is still possible though with a much reduced frequency in the embryonic phase. It is very difficult to induce ESM formation beyond the embryonic phase.

Somatic embryogenesis systems have been developed for a large number of conifer species in research laboratories all over the world and they all tend to follow the same pattern. Explants are treated with media containing a relatively high concentration of 2-4Dichlorophenoxyacetic acid (2-4D) to induce the ESM. This is then grown on a maintenance medium, also containing 2-4D. The ESM tends to be very fast growing so it needs to be subcultured at frequent intervals of between two and four weeks. The ESM produces the earliest stages of embryo formation. To mature the early stage embryos, the ESM is transferred to a medium containing abscisic acid. This triggers the development of the embryos into mature embryos that are capable of

germination when transferred to a hormone free medium. The germinated embryos can then be transferred to soil.

Somatic embryogenesis is the propagation technique that offers most promise for clonal forestry. It has the largest potential multiplication rate and the plants it produces are always juvenile. It is expected that the growth and development of somatic seedlings would mirror that of zygotic seedlings. Most importantly, ESM can be cryopreserved. Plants can be generated from the ESM whilst a portion of the ESM is held in cryostorage. The plants can be put through a normal genetic field test and good clones identified. These good clones can then be generated in the juvenile form from the cryostored ESM and used in clonal forestry. This fulfils all the requirements of the definition of clonal forestry given at the start of this paper.

The major problem with the *in vitro* techniques is they are very labour intensive and therefore produce plants that are very expensive compared to normal seedlings. Is it possible to introduce some form of automation into the various techniques? There is already a degree of robotics in media preparation, cutting of microshoots, subculturing and planting in soil using already existing machinery but it can be seen that much very specialised and sophisticated machinery will need to be developed to do the complex tasks needed in micropropagation and somatic embryogenesis.

A relatively simple way of semi-automation might be to transfer technology from microbiology and use bioreactors. They are attractive because pH, oxygen, carbon dioxide, shear forces, temperature and light quality can be controlled and the various stages such as media changes can be done automatically and the amount of manual handling is dramatically reduced. ESM of a number of conifer species have been has been grown in bioreactors. The tissue grows very well but only in the maintenance phase. It has proved very difficult to mature the ESM to form mature somatic embryo in the bioreactor. There have been no published reports of this having been done. It is probable that bioreactors will be used in somatic embryogenesis but only to produce large amounts of ESM very quickly. The mature embryos will subsequently be formed on some sort of agarised or semisolid medium.

A technique that is currently being investigated is that of temporary immersion. The ESM is held on an inert support and nutrient medium containing various control chemicals pumped into the chamber for a time and then drained away again. The process can be fully automated and treatment solutions easily changed as necessary. This technique appears to get around the problem of non-maturation in bioreactors and is an exciting prospect for the future.

The new technologies will have a dramatic effect on the selection and breeding of conifers and it is from this that clonal forestry will emerge. In a typical breeding programme, a base population of plus trees is subjected to half-sib progeny testing and from the results a breeding population can be created. This breeding population can be further refined by assortive mating and full sib progeny testing. A production

population can be established from the breeding programme and seed orchards established to produce family mixtures that can be vegetatively multiplied in a cuttings programme to yield improved material. It might be possible to take mature clones from the full sib progeny tests, rejuvenate it and the rejuvenated material used to produce improved clonal material. However, it seems that rejuvenation might not be an option at the moment.

Another path to clonal forestry would be to take full sib seed from the assortive mating of the breeding programme and establish it *in vitro* as embryogenic tissue. Some of this could be cryogenically stored and some genetically tested. The good clones could then be used for the production of plants for clonal forestry.

The technique that will have the most dramatic effect on tree breeding and clonal forestry is marker aided selection. If genetic markers can be identified for a range of traits, the requirement for the field testing of genetic crosses will no longer be necessary. It is likely that this will be in the far future as a vast amount of research is necessary before the technique can be used. A large number of trees will need to be screened to find the required markers and the traits present compared to the markers present or absent. It will then be necessary to test the markers in a range of progeny tests to get absolute correlation between the traits present and the genetic markers present. It is only then that genetic markers will be able to be used as the predictors of performance of any juvenile genotype.

It is possible to construct a scenario of what some of this new technology will allow us to do in the far future. Trees will be screened in the forest or in progeny tests for genetic markers that will demonstrate that they will pass on their desirable characters to their progeny. Controlled crosses will be made and embryogenic tissue established from the immature embryos. The embryogenic tissue could then be screened for a whole range of desirable and undesirable characters. The selected clones could then be cryopreserved until required. When required, the clone could be withdrawn from cryogenic storage and processed to form mature embryos and subsequently, artificial seeds. The artificial seeds could then be sown in seedbeds and when germinated, managed as we manage seedlings today. The plants would then be used to establish clonal forests.