# Aims and Results of Basic Research in the Institute of Forest Tree Breeding in Waldsieversdorf, Germany

## I. The Development of Biotechnological Research

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**Abstract**

The paper reports the development of micropropagation methods and progress using enzyme gene markers for practical forest tree breeding. It covers recent work in these fields at the Institute of Forest Tree Breeding. Micropropagation methods have been developed to support practical forest tree breeding by multiplying selected clones of several deciduous trees (e. g. birch and aspen) for the establishment of field trials.

Coniferous tree species (larch, Douglas fir and Norway spruce) have several species-related advantages and disadvantages concerning their micropropagation behaviour. Therefore, one important task of biotechnology is to utilize experiments to develop research strategies that will solve problems of plant regeneration.

Gene markers based on isoenzyme analysis are able to provide information about the genetic structure of stands and breeding material. Using these methods, investigations were carried out with Norway spruce, Scots pine, larch, Douglas fir and beech concerned with problems of hybrid identification and the conservation of gene resources.

*Key words:* Forest tree breeding, tissue culture, micropropagation, enzyme gene markers, Norway spruce, larch, Douglas-fir, Scots pine, beech.

Since the end of the 1970s, efforts have been made to introduce biotechnological methods as a tool of vegetative propagation at the former Institute of Forest Sciences Eberswalde, Department of Forest Tree Breeding, Waldsieversdorf. Based on available knowledge, first attempts resulted in a spontaneous organogenesis of some birch clones.

However, some of these early experiments showed that higher concentrations of plant growth regulators used for the subculture of calli sometimes led to shoot cultures which became incapable of further progress toward organ formation (e. g. root formation). This was one of the key findings that influenced all subsequent research in this field. In the early 1980s a research group for biotechnological research was founded. The first projects were (1) the development of methods for micropropagation of several curly birch clones (*Betula pendula f. carelica*) derived from breeding programmes, and (2) the search for tissue culture methods to multiply aspen and hybrid aspen clones (*P. tremula* x *P. tremuloides*) selected for air pollution tolerance and resistance to snow breakage (NAUJOKS et al., 1987; EWALD et al., 1991). The vegetative propagation of mature birch and aspen trees via woody or green cuttings was difficult (e. g. birch trees need a total crown reduction to form new shoots with the ability to root).

To ensure that the regeneration of birch from organogenic calli did not influence plant morphology, several birch mutants with different morphological patterns (leaf structure, colour) were included. Of the thousands of plants regenerated, none showed any morphologically different plants or stable mutants (MATSCHKE et al., 1987).

Explants derived from donor trees of birch and aspen up to age 40 reacted without any remarkable difference in propagation or rooting behaviour after establishing clone lines.

Clonal differences in propagation rate were much more important. The distribution of aspen clones with regard to propagation rate was nearly the same as that of AHUJA (1983). One fourth of the clones were economically feasible to propagate, one half grew slower but sufficiently rapid to obtain plants for field trials. The last fourth were difficult to propagate at all.

While propagating large amounts of plants (*Figure 1*) we were confronted with problems not encountered in the development of laboratory methods (e. g. root development and transfer to soil) but of enormous significance to the later growth of these plants in the nursery and forest.

We were therefore, forced to optimize bioregulator concentrations, for example for root formation, in order to

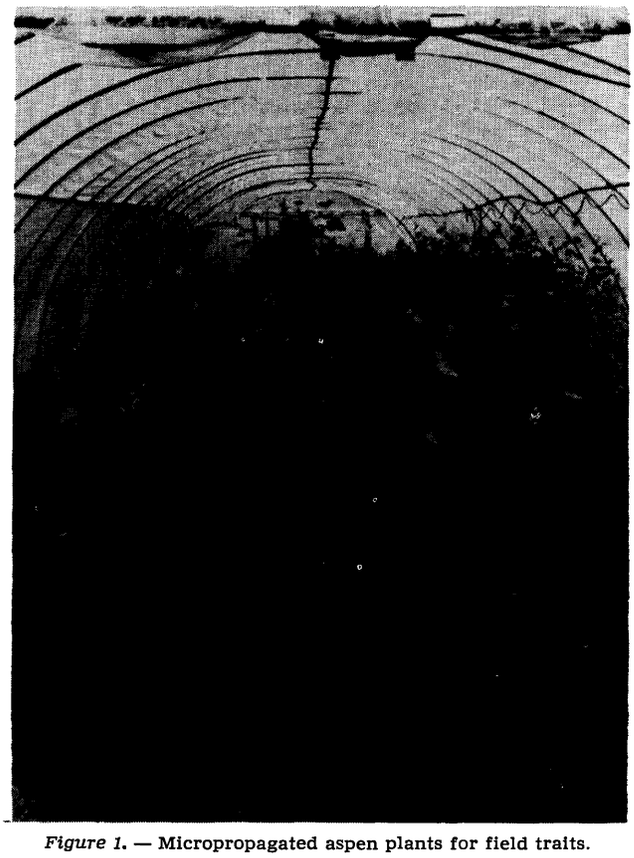

*Figure 1.* — Micropropagated aspen plants for field traits.

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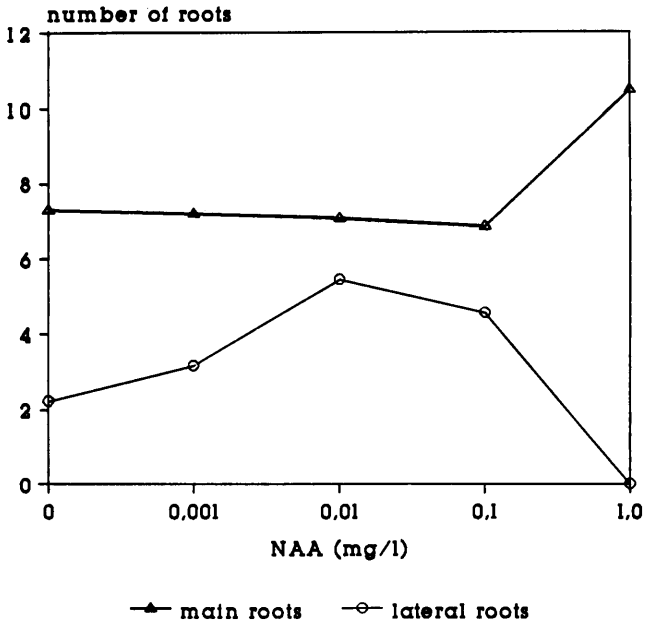


clone Bä 13.
18 days after transfer on NAA containing media

*Figure 2.* — Influence of naphthaleneacetic acid (NAA) concentrations on number and branching of formed roots in aspen.

focus on quality as well as quantity. *Figure 2* shows the influence of the auxin NAA on the development of lateral roots. The enhancement of auxin concentration led to higher numbers of main roots but diminished the formation of important lateral roots.

In close cooperation with horticultural enterprises, our laboratory provided clones for mass propagation and the establishment of field trials. The tests of one enterprise (HAENTSCH unpubl.) to compare the real propagation factors for 5 curly birch clones over longer periods with the calculated theoretical ones showed the limited value of such calculations (WHITEHEAD and GILES, 1977).

Since 1986, the main task for biotechnology has been the development of methods for conifer micropropagation. The investigations had the objective of increasing knowledge concerning the physiological regulation of differentiation and organ formation. It was felt that with the support of biotechnological methods the shortening of breeding periods required to multiply plant material (e. g. seeds) for later vegetative propagation could be achieved. Plant materials chosen, for which methods would be developed, were (1) hybrids of Douglas-fir and larch that were tolerant of air pollution, and (2) Norway spruce taken from heavily-damaged populations in the Saxon Ore Mountains. The relative high growth potential of Douglas-fir and larch seedlings under red light led to the creation of a propagation method that used axillary bud-bearing segments (*Figure 3*). The method was developed in close cooperation with a research group at the Humboldt University in Berlin (HÜBL and ZOGLAUER, 1991). Broad experimental series were carried out for all factors that could improve the individual steps in propagation. Some main factors which influenced the growth of conifers were estimated.

The influence of different light qualities (white, blue, red) on shoot growth was tested, and led to the above-cited conclusion that red light had the most stimulating effect on shoot growth.

By testing the effect of different nitrogen sources, optimum systems for larch, Douglas-fir and Norway spruce have been developed. In larch, for example, glutamine was most successful for shoot growth stimulation, in Douglas-fir shoot growth was inhibited by ammonia ions. For phytohormone-mediated induction steps leading to bud or root formation, concentration of the basal medium and the nitrogen supply were as important for the number and later development of formed organs as the phytohormone concentrations used. A frequently-reported negative correlation between the number of buds induced and elongation was usually observable. This necessitated to long-term test systems to get useful plant material (rootable shoots). The evaluation of propagation parameters over several years showed different propagation rates and degrees of rooting with the same clonal material. These results have been often in contradiction with the expected results based on mathematical calculations from

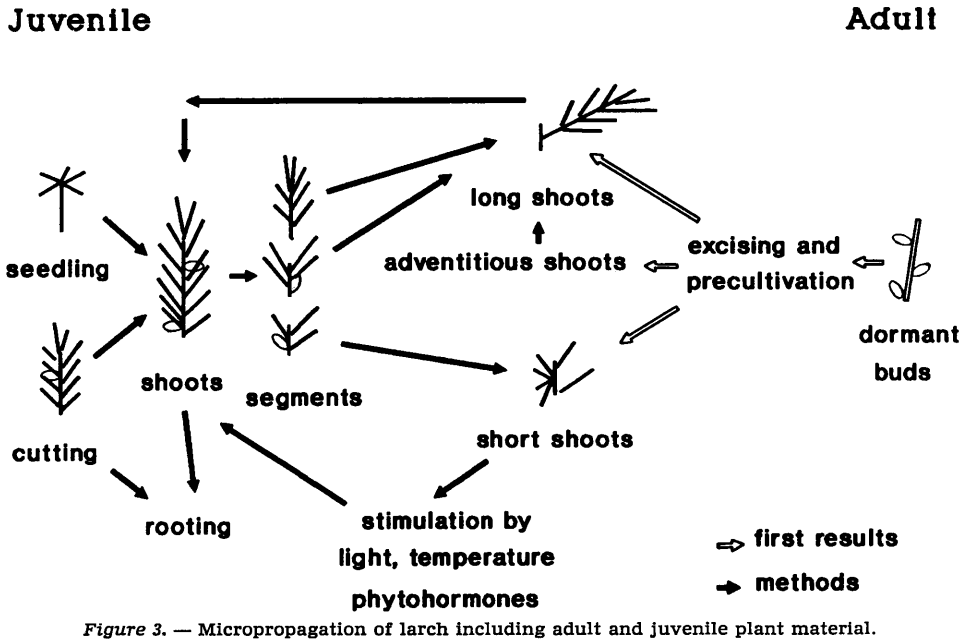


*Figure 3.* — Micropropagation of larch including adult and juvenile plant material.

*Figure 4.* — Plants of hybrid larch derived from micropropagation.

experiments. Methods that have been developed with ordinary seed material in many cases had to be modified for selected seed material. The hybrid seeds originating from controlled pollination between green and blue varieties of Douglas-fir had a propagation behaviour which was different from that of the seed material with which the methods were developed. This concerned both the poorer elongation capacity and the much more difficult rooting behaviour and transfer to the soil.

Propagation methods developed earlier for Douglas-fir and larch (*Figure 4*) also influenced the research with spruce. In order to develop a uniform axillary bud-bearing system, all factors with a possible influence on growth velocity were tested, including known, new and artificial phytohormones, nitrogen supply and light conditions.

The conflict between shoot elongation and lateral bud formation in juvenile and "fast" growing Norway spruce shoots was a physiological barrier that we could not overcome.

This experimental background showed that every species has its own advantages and disavantages for in vitro propagation (organogenesis) of juvenile plant material (*Tables 1, 2* and *3*).

On the basis of our knowledge of difficulties and problems occuring during certain stages of organogenesis we directed our attention to the particular problems that prevented effective propagation of each species. For larch it was short shoot formation, for Douglas-fir the stimulation of lateral bud formation and subsequent development and also the poor root formation, and for Norway spruce we tried to improve the processes of adventitious shoot formation.

*Table 1.* — Shoot elongation capacity of larch, Douglas-fir and Norway spruce in vitro.

| Shoot elongation | larch | Douglas-fir | Norway spruce |
|---|---|---|---|
| shoot growth | fast<br>25 mm/month | fast<br>16mm/month | slow<br>6mm/month |
| occurrence of lateral buds in relation to the elongation of shoots | independent | depending on genotypes | the faster the shoot elongation the lower the number of lateral buds |
| elongation of lateral buds | diminished by short shoot formation | depending on genotypes | normal |
| basis for a micropropagation system (via shoot segments) | yes | in general yes | no |

*Table 2.* —Advantages and disadvantages of adventitious bud formation for larch, Douglas-fir and spruce in vitro.

| adventitious bud formation | larch | Douglas-fir | Norway spruce |
|---|---|---|---|
| induction of adventitious buds | occurrs - difficult to separate (bud) clusters | negative correlation of bud number and later elongation | negative correlation of bud number and later elongation |
| elongation behaviour | elongation of shoots depends on genotypes used | fast elongation, diminished after repeated cycles of segment propagation | diminished after repeated induction cycles |
| process repeatable (continuous) | yes | unknown | yes |

*Table 3.* — Root formation capacity of larch, Douglas-fir and spruce in vitro.

| root formation | larch | Douglas-fir | Norway spruce |
|---|---|---|---|
| spontaneous root formation on shoots | rare | no | yes |
| period of root formation after root induction | short (6-10 weeks) | long (>15 weeks) | short (6-12 weeks) |
| quality of induced roots | good (branching of roots) | poor (low number of lateral roots) | good (branching of roots) |
| problems | plagiotropic growth after transfer to the soil (1-2 years) | transfer to the soil, long lasting plagiotropic growth (for several years) | difficult formation of roots on shoots derived from repeated shoot induction steps |

Our results showed that the stimulation of shoot elongation in short shoots of larch was possible by a combined treatment with cytokinins that included a modification of physical parameters (light and temperature). By this treatment more than 50% of short shoots developed into long shoots (*Figure 5*).

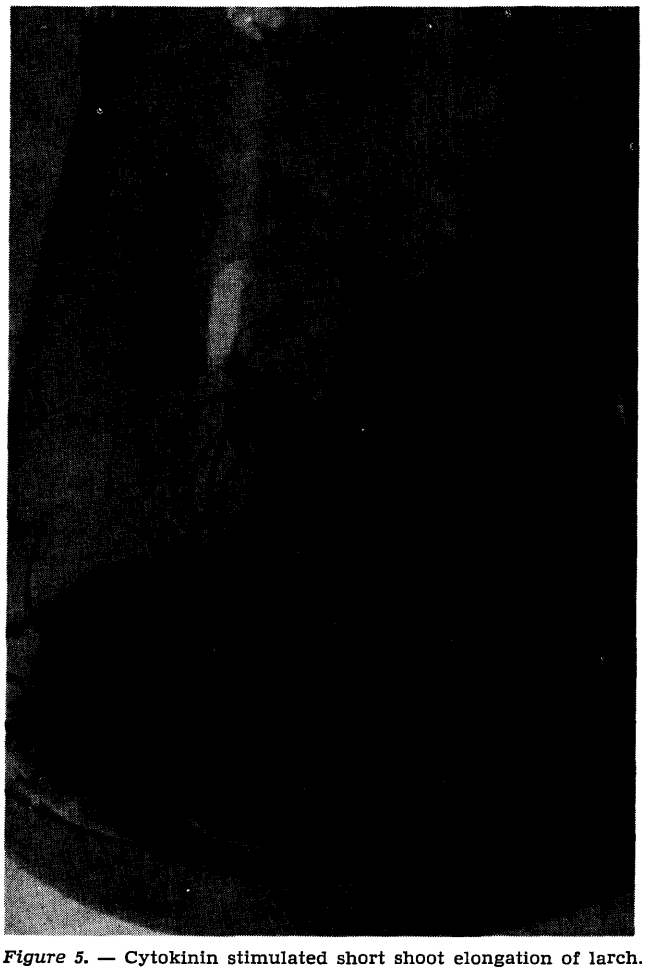

*Figure 5.* — Cytokinin stimulated short shoot elongation of larch.

In Douglas-fir experiments it was also possible to increase the number of lateral buds per shoot and to induce the breaking of these buds by cytokinin treatments. Cytokinin treatments of shoots 8 weeks before root induction improved the percentage of later root formation. The short shoots had been propagated for several months by methods described above. This is an example of the importance of cytokinins in conifers as well as deciduous trees during the process of root formation.

Nevertheless, micropropagation of Douglas-fir is still limited by unsatisfactory root formation and later plagiotropic growth behaviour.

In Norway spruce experiments, the search for factors stimulating the elongation of induced adventitious buds also led us to the conclusion that the bud induction process is responsible for the later development of the buds formed (*Fig. 6*). The high number of buds formed after repeated induction steps alternating BAP with phases free of phytohormone influence is closely correlated with the number of dormant buds and their inability to break. The presence of native cytokinins in lower concentrations allowed us to multiply adventitious buds (*Fig. 7*) including their later elongation (EWALD, unpubl).

This search for optimizing processes of organogenesis with juvenile plant material is one necessary precondition for the work with adult plant material.

The preconditioning of adult plant material is also important, in order to stimulate the developmental processes of plant material used for in vitro culture.

The use of phytohormone-free preculture of winter buds from 140-year-old larch trees before phytohormone stimulation resulted in elongation of short shoots of one clone. The growth rate of these shoot cultures was sustained for more than 8 months and was similar to comparable cultures derived from seedlings (KRETZSCHMAR, unpubl.). This confirmed the hypothesis that establishment of fast-growing shoot cultures is possible with the use of mature plant material.

The preconditioning of mature trees includes all methods of rejuvenation, such as grafting on a juvenile rootstock, hedging and several other methods influencing the mature tree.

Our own attempts to develop micrografting methods for larch and spruce showed that choice of the rootstock (e. g. seedling or rooted hypocotyl cutting) and the physiological development of the bud or meristem used

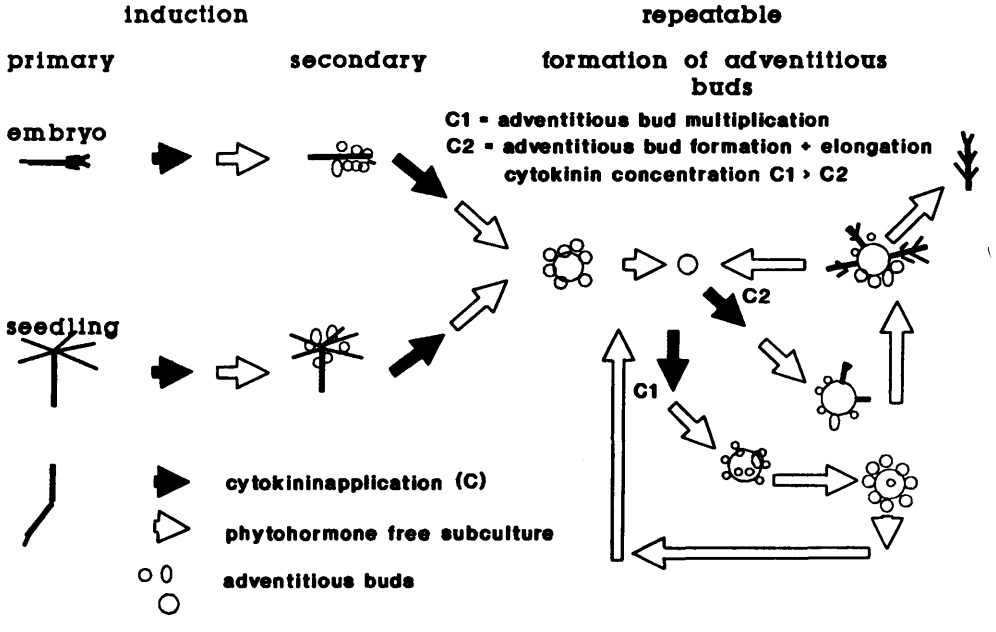


*Figure 6.* — Phases of adventitious bud formation on juvenile spruce explants.

*Figure 7.* — Adventitious shoot clusters of Norway spruce.

(e. g. winter buds or newly formed buds after flushing) were the factors mainly responsible for grafting success.

Changes of needle parameters (e. g. width) in grafted spruce buds confirmed similar results that were obtained with other conifers (EWALD et al., 1991). Further experiments are needed to test the influence of the juvenile rootstock on morphological and growth parameters of grafted organs.

Organogenesis has not been the only research interest during the last few years. In close cooperation with other scientific institutions, methods have been developed for the establishment of embryogenic lines of spruce (SÜSS et al., 1990) and larch, including early stages of plant regeneration. These first encouraging results elucidated to some extent the processes involved in plant regeneration via somatic embryogenesis in spruce and larch. The results demonstrated the developmental regulation is a delicately-controlled phenomenon in conifers. Additional research work must be done on the processes of both somatic embryogenesis and organogenesis in conifers to more fully understand it and to carry out an effective plant regeneration in vitro.

**Literature**

(see part II).

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## II. The Use of Enzyme Gene Markers for Practical Breeding Tasks

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Modern concepts for conservation of the genetic resources of forests and for breeding methods need support from genetic research. For more than 20 years, the use of enzyme gene markers in forestry has been a successful way of acquiring information on the genetic structure of provenances, populations and individual trees.

In the last few years, molecular genetic approaches, such as analysis of restriction fragment length polymorphisms (RFLPs) and DNA fingerprinting, have been applied to forest genetics, opening new possibilities for studies of the nuclear and organelle genomes. The older, well-tested method using isoenzyme markers, can still be applied to research on population genetics and breeding and is used in many institutes. A discussion of our work using isoenzyme methods follows.

Proteins were extracted from haploid (conifer endosperm) or diploid (buds, leaves, callus) tissues by homogenization in an extraction buffer. After the electrophoretic separation of proteins in a polyacrylamide or starch gel, the enzyme proteins were specifically stained. The isoenzyme

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