CHAPTER 12

MICROPROPAGATION OF *LARIX* SPECIES VIA ORGANOGENESIS

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1. INTRODUCTION

Larch is a conifer which is characterised by relatively fast growth. Among conifers, its specialised trait of losing its needles in the winter has some advantages, especially in areas with high levels of air pollution. The breeding history of this tree species in Europe, especially in Germany, goes back more than 40 years. Today several possible parent combinations are known, seed orchards are established, and field trials and natural stands exist, which allow the selection of suitable material for practical purposes as well as for continued breeding and tree improvement. Some of the selected tree stocks are already characterised according to their wood quality and resistence to decay. Larch is known as a wood which normally does not need any chemical protection. This will make larch of increasing interest for forestry in the future. Conifers often flower well only at intervals of several years, in an irregular cycle. As a result, seed material derived from seed orchards and from controlled pollination is not available every year and often is only available in limited amounts. This situation has resulted in the search for vegetative and microvegetative propagation methods for larch. Tissue culture and micropropagation methods were evaluated for these reasons. Seedlings from hybrid larch, characterised by a faster growth rate and an increased tolerance to air pollution, were used to investigate these methods. Larch clones are important for research tasks (e.g. resistance research) and for establishing clonal mixtures suitable for reforestation. Regeneration systems in vitro are necessary preconditions for gene transfer as well. Therefore the basic methods were developed and established for juvenile plant material (zygotic embryos, seedlings, saplings). Foresters, however, are generally interested in trees that have proven quality traits such as growth performance and resistance over long periods. This assessment is often

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made at half the rotation age. The negative aspect of such an extended assessment is that, by that time, most of the individuals have lost their ability to be propagated vegetatively. Moreover, even when vegetative propagation of selected adult individuals is possible, it is often linked with improper root formation and plagiotropism. For different *Larix* species, there is interest in obtaining propagules with juvenile growth behaviour from selected adult trees. This includes trees from natural stands as well as hybrids derived from breeding experiments. But micropropagation of mature trees is often, although not always, more difficult than *in vitro* propagation of juvenile material such as zygotic embryos or seedlings. This is especially true for some of those conifers used in large scale forestry, including larch (Bonga & von Aderkas, 1988; Chalupa, 1991, 2004; Karnosky et al., 1993). Plant production from shoot formation or shoot development is often lower than from cultures initiated from juvenile plant material. Cultures of adult and juvenile origin also differ in growth behaviour and morphology. Finally, both rooting success and transfer to the soil pose problems because of reduced root growth. Most of the difficulties are due to phase changes during tree development. Nevertheless, it is reportedly possible to overcome these difficulties, at least for those genotypes which showed better responses to tissue culture (Bonga & von Aderkas, 1993). Preconditioning of the plant material (grafting, pruning) is sometimes required and different tissue culture methods must be optimised to gain a degree of reinvigoration and rejuvenation of the plant material. Attention was also focused on possible factors responsible for successful propagation of adult donor trees. This chapter will summarise the work accomplished to date, and will consider possibilities and problems for future work.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Supply of Plant Material

Juvenile plant material. The plant material (seeds) for the development of propagation methods – either from seed orchards or derived from controlled pollination – was provided by different breeders from Brandenburg and Saxony, but also from Russia and China. Various larch species and hybrids have been included in the experiments over the last 20 years: *Larix eurolepis*, *L. decidua*, *L. kaempferii*, *L. sukaczevii*, *L. gmelinii* and others. Especially hybrid larch trees (*L. eurolepis*) characterised by a higher growth performance and frost tolerance were selected for experiments. Elongating shoot tips as well as long-shoot buds of 1- or 2-year-old plants from the nursery were used to establish tissue cultures.

Adult plant material. From adult donor trees, it was possible to use closed winter buds harvested in autumn after needle fall, because this allowed effective disinfection and preparation. Furthermore, good growth of buds and meristems (e.g. for micrografting) was achieved because material was not yet in deep dormancy. Micrografting experiments and attempts to induce adventitious buds demonstrated that this was the best time for establishing cultures from terminal long-shoot buds.

2.1.2. Disinfection of Plant Material
Seeds. Seeds were disinfected for 10 min using mercuric chloride solution (0.25%) containing a drop of detergent (e.g. Tween 80). They were then rinsed three times with sterile deionized water and placed onto a nutrient medium free of phytohormones for germination (BEMB/200). In one case, when seeds of *L. gmelinii* were used, this method failed because of the heavy infection with fungi in the seed coat. Seeds were rinsed for 1 min in 70% ethanol and the seed coat was then removed with a scalpel. The megagametophyte containing the embryo was placed directly on germination medium or was used after a second but shorter disinfection period (3–4 min) with mercuric chloride.

Growing shoot tips/winter buds. Growing shoot tips as well as winter buds from the nursery were sometimes heavily infected, especially during wet weather periods. Thus, if possible, the plants were potted and placed in a greenhouse before taking explants. Treatments with fungicides a few days before harvest of plant material improved the disinfection success. The explants always had a dry surface before disinfection: the method was identical to that used for seeds. Before placing explants on medium, the shoot base was removed with scissors. Used mercuric chloride solution was collected and disposed of as hazardous waste. Mercury could be precipitated after the addition of ammonium disulfide solution and the excess water could then evaporate under an extractor hood. Solid precipitated mercurysulfide was disposed of by specialised enterprises.

2.2. Culture Media

The composition of culture media for larch micropropagation is described in Table 1, modifying the following basal plant nutrient media.

MCM (modified, urea lacking), according to Bornman, C.H. 1983
BEMB (modified,) according to von Arnold, S. & Eriksson, T. 1981
(macroelements), and Boulay, M. 1979 (microelements)
B, according to Boulay, M. 1979
L9 based on L according to Linsmaier, E.M. & Skoog, F. 1965.
Wz based on WPM according to Lloyd, G. & McCown, B. 1981.
SH according to Schenk, R.U. & Hildebrandt, A.C. 1972.

Growth regulators which were supplemented are mentioned seperately in the text. All media were solidified with agar or gelrite.

2.3. Shoot Regeneration and Maintenance

2.3.1. Serial Propagation of Juvenile Explants
The organogenic potential of plant parts from larch seedlings can be used for several tissue culture propagation methods. The two possibilities described in the following are serial subcultures without phytohormones and adventitious bud formation. There

Table 1. *Basal nutrient media compositions used for larch micropropagation (macroelements, microelements given as dilution of the original medium).*

Medium	Macro-elem.	Micro-elem.	Carbon source $g\ l^{-1}$	NH_4NO_3 $mg\ l^{-1}$	PVP $mg\ l^{-1}$	Arg $mg\ l^{-1}$	Gln $mg\ l^{-1}$	pH
MCM	1/2	1/2	15 S		100	100		6.8
Wz gluc	1	1	32.87 G					5.7
BEMB/ 200	1	1 (B-med.)	10 S	200	200			5.8
BEMB/ 600	1	1 (B-med.)	10 S	600	200			5.8
B1	1	1	30 S				146	5.7
L9	1/3	1	5 S					5.7
SH 1/2	1/2	1/2						5.7

PVP – polyvinylpyrrolidone, Arg – arginine, Gln – glutamine, S – sucrose, G – Glucose

are only a few publications which discuss use of phytohormone-free media to induce organ development in larch. Most of the authors tried to stimulate axillary bud development or to induce adventitious buds by phytohormone treatments (see 2.3.3. Adventitious bud formation). By exploiting the capacity for shoot elongation in larch, Douglas fir and Norway spruce, it became obvious that larch had a higher potential for shoot elongation on a phytohormone-free medium compared with Douglas fir or Norway spruce. This was the background used to develop the so-called "serial propagation", which was carried out according to methods developed for juvenile larch shoots (Hübl & Zoglauer, 1991).

Culture conditions. Serial propagation without phytohormones is based on the continuous growth of elongating larch shoots *in vitro* on B1-medium at a temperature of 23°C. The illumination condition was continuous red light (fluorescent tubes OSRAM L58 W/60, red; 30 µE $m^{-2}s^{-1}$, 650 nm peak emission), which was found to force shoot elongation much better than blue or white light.

Dividing of elongated shoots into segments. Once the shoot axis of the sterile germinated seedling or of a long-shoot bud from a juvenile plant had elongated (Figure 1A), the shoot was cut into a shoot tip and bud bearing stem segments (appr. 15 mm long) which were transferred to fresh B1-medium, where they sprouted and formed long-shoots again.

Subcultures. Subcultures were carried out in 7-week-intervals in tubes or Erlenmeyer flasks. On such an elongated larch shoot, those buds located close to the shoot base are determined to become short-shoots. As in many other tree species, establishment of a propagation culture required a few subculture intervals until optimal propagation factors were reached.

Stimulation of elongation. Shoots showing a larger number of lateral buds with failing bud elongation were treated for 4 weeks with the nutrient medium normally used for adventitious bud induction (MCM + zeatin/kinetin – see 2.3.3.). The medium Wz gluc, containing 1.5 mg l^{-1} zeatin in combination with glucose as a carbon source, was used in the same way to stimulate bud elongation. After this step, the shoots were placed again on the normal elongation medium B1. In many cases, the buds started to sprout shortly after transfer to the phytohormone-free medium. Beyond a certain bud size the inducing effect of the adventitious bud induction medium changed into an elongation-stimulating effect.

Short-shoot stimulation on juvenile explants. With increasing age a larch seedling in the field forms more and more short-shoots. This means that not all buds are able to form an elongating shoot, a long-shoot. Short-shoot buds are characterised by a smaller meristem, which is not as long as the preformed needles, in contrast to a long-shoot meristem. Short-shoot buds form only needle clusters. Terminal buds of larch usually contain long-shoot meristems. The two or three lateral buds below the terminal bud often contain long-shoot meristems as well, whereas the next lower two or three buds can be characterised as intermediate forms of meristems with an increasing tendency to express short-shoot meristems.

In cases where the terminal bud was lost, the next shoot meristem was capable of developing an elongating shoot axis. In juvenile material (4-month-old seedlings) more buds are able to form long-shoots. In older material and especially in old-aged cultures, short-shoot buds refuse to form a long-shoot if the shoot axis is cut into bud bearing stem segments. Therefore an *in vitro* method was developed to stimulate sprouted short-shoot buds to elongate a shoot axis by a combined treatment of cytokinins, light and temperature (Kretzschmar, 1993).

Induction of short-shoots. Non-growing short-shoots were incubated in 100 ml-Erlenmeyer flasks on half strength MCM medium (Table 1). Kinetin (0.5 mg l^{-1}) and 0.05 mg l^{-1} indole-3-acetic acid (IAA) were added as growth regulators. During the induction treatment, the cultures were kept at 17°C under a photoperiod of 16 h in white light (radiation 30 μE m^{-2} s^{-1}) for 4 weeks.

Elongation of short-shoots. After that treatment, the explants were transferred onto the elongation medium free of phytohormones (B1) under continuous red light at 23°C. After 8 weeks, up to 63% of short-shoots elongated and could be used for serial propagation again.

2.3.2. Serial Propagation of Adult Material

The method of serial propagation as already described was also applied to very old material (from over 40 years up to 120 years in age). From 42 clones established that way, it was possible to propagate only 4 lines (Kretzschmar & Ewald, 1994; Ewald & Naujoks, 2000). Lack of elongation capacity in long-shoot buds was still the limiting step. From the established clone lines, three clones were rooted sucessfully. It was possible to improve the elongation capacity of shoots by a subculture on nutrient

medium containing a cytokinin, based on the experience of short-shoot stimulation in larch described already for juvenile material. The nutrient medium Wz gluc supplemented with 1.5 mg l^{-1} zeatin was used for this subculture. Glucose as a carbon source was sometimes more efficient in other trees as well (G. Naujoks, personal communication 2003; e.g. for oak). For different recalcitrant tree species, this medium was used for induction as well as for organ development. After such an intermediate step the elongation of shoots was forced.

2.3.3. Adventitious Bud Formation – Juvenile Material

Zygotic embryos, germinating seedlings, and shoot tips as well as winter buds were used for the induction of adventitious bud clusters. Zygotic embryos were removed from the endosperm after one day of germination.

Induction of adventitious buds and subculturing. After disinfection, all explants were placed directly on MCM medium (Table 1) supplemented with 1.5 mg l^{-1} zeatin and 0.15 mg l^{-1} kinetin for 4 weeks. Within the following two subcultures (7–8 weeks) clusters of adventitious buds developed on a medium without plant growth regulators (BEMB/200). One propagation cycle consisted of the induction period and two subcultures for the development of induced buds. Afterwards, the bud clusters were divided into single explants. These explants were used for a new cycle. All subcultures were carried out under continuous red light at 23°C.

Elongation of shoots. Shoots with an axis longer than 10 mm after at least three cycles were cultured on a medium without plant growth regulators, but with an enhanced concentration of ammonium nitrate (600 mg l^{-1}; BEMB/600) in order to support shoot elongation. After shoots had reached a length of 30 mm, they were transferred to standard cultivation medium B1 (Ewald et al., 1997).

2.3.4. Adventitious Bud Formation – Adult Material

Induction of adventitious buds and subculturing. The induction and development of adventitious bud clusters was carried out as described with juvenile material, but using long-shoot buds in October. Physical culture conditions were identical to juvenile material. In some cases the very first induction medium was supplemented additionally with 0.5 mg l^{-1} benzylaminopurine (BAP) to increase the number of buds formed. In the following cycles BAP was omitted to avoid the inhibition of shoot elongation, as had been observed in experiments using BAP for repeated induction steps with different conifers (larch, spruce, yew). The establisment of a well propagating culture of adventitious bud clusters of adult larch continued for a period of at least 2 years (appr. 8 propagation cycles). In the following period, the medium Wz gluc (+1.5 mg l^{-1} zeatin) was used to induce buds. In this way the elongation potential could be enhanced.

Elongation. The beginning of the elongation of adventitous buds was achieved, and was visible as a 1–2 mm stem-like region at the base of buds (Figure 1I). This occured after a complete propagation cycle (12 weeks) followed by repeated phytohomone-free subcultures (BEMB/200). Spontaneous rooting was sometimes observed in these

little shoots (Figure 1J – see also 2.4.2.). Such shoots were the basis for ongoing experiments to induce a controlled elongation comparable to the short-shoot stimulation method mentioned before.

2.3.5. Preconditioning of Adult Plant Material (Micrografting)

The micrografting procedure consists of grafting into the top of the sprouting stem axis (epicotyl) after removal of the shoot tip of a very young seedling *in vitro* (Ewald & Kretzschmar, 1996). The method was carried out with disinfected seedlings grown directly in Jiffy-7 peat pellets (∅ 38 mm, JIFFY Products Ltd., Norway) in a petri dish. When the epicotyl stem axis of the seedlings reached about 20–30 mm, the upper 10 mm was exised and discarded and the remaining portions of the seedlings were used as rootstocks. It was important to use a sterile or semisterile system to prevent fungal infections of the grafted meristem as well as of the surface of the grafting union. Larch seeds were disinfected as already described. Jiffy peat pellets were fully saturated with deionized water and were autoclaved three times in intervals. Afterwards two peat pellets were transferred into one sterile petri dish (105 mm in diameter). Germinating seeds were placed directly into these peat pellets. Because a minimum length of at least 20–30 mm of the newly formed stem axis is necessary, a longer germination period (appr. 12 weeks starting in June) had to be calculated. Twigs (30 mm) of adult selected donor trees were harvested in October and treated with a fungicide (e.g. 0.2% Euparen by BAYER, 50% dichlorfluanide, w/v) one day before use. Twigs were disinfected as already described. The grafting procedure was carried out under a stereomicroscope in a laminar flow box. To prevent dehydration of the meristem as well as of the cleft of the rootstock, a step-by-step system was developed to make rapid grafting possible. Two kinds of scalpels were used: normal scalpels with replaceable blades and special, extremely sharp small scalpels made of razor blades (handled with a holder) to cut out the meristem and to transfer it. All blades were disinfected during the grafting procedure by wiping them on a wet sponge saturated with 0.4% peracetic acid solution. The petridish with the rootstock was opened briefly and needles along a 15 mm length of the green elongating part (grafting area) of the seedling were removed with a scalpel.

1) The twig with the terminal long-shoot bud (Figure 1D) was placed under the microscope. The budscales were removed with a normal scalpel and discarded. A horizontal cut in the direction of growth near the meristem provided an even surface. A parallel cut behind the meristem counter to the direction of growth provided an area in which the meristem was situated.
2) The rootstock was placed under the stereomicroscope again. The shoot axis was cut and a cleft of approximately 3 mm was made in the top with the razor-blade-scalpel.
3) The prepared twig with the precut meristem was placed under the microscope again. Two wedge shaped cuts were made in opposite directions to cut out the apical meristem complex (like a piece of cake – about 0.3–0.5 mm in diameter). Dissected tissue included the apical dome and the first ring of needle primordia to prevent desiccation of the meristem after insertion. The first cut was made on

the left and the second on the right side. The meristem was taken out with the left side of scalpel blade to avoid gripping and damaging it with forceps (Figure 1E).

4) Afterwards, the meristem was subsequently fixed onto the blade side for transfer and placed in the cleft cut within the rootstock, using the blade to open the cleft and strip off the meristem.

The root system was not disturbed during or after the grafting process because the grafting was carried out within the petridish. All rootstocks and micrografts were cultured at 23°C under continuous red light conditions. After formation of a graft union, the grafts were potted with the peat pellet and transferred to the greenhouse for weaning (Figure 1F). After sprouting of shoots and development of plants, all micrografts should be checked by suitable isozyme analysis. Undetected adventitious bud formation from rootstock material occurred frequently.

Cutting propagation. After 4 years, cuttings taken from micrografts and rooted in June/July with a 2 g l^{-1} indolyl butyric acid (IBA) containing rooting paste (Figure 1G) resulted in fast growing plants 5 years later. This was impossible directly from the adult donor trees. These plants behaved like seedlings (Figure 1H) and did not show any precocious flower formation.

2.4. Rooting of Shoots

2.4.1. Juvenile Material

Root induction after serial propagation. Newly formed shoot tips, approximately 30–40 mm long and without any visible bud primordia, were subcultured for root induction on L9-medium supplemented with 2 mg l^{-1} naphthalene acetic acid (NAA) for 2 weeks. The induction by use of NAA was found to be more beneficial than that with IBA (Dembny & Zoglauer, 1992). During root induction and development, the temperature was reduced to 17°C and the photoperiod consisted of 16 h white light (OSRAM L58 W/31-830).

Root development. After 2 weeks, the induced shoots were transferred directly into Jiffy-7 peat pellets (∅ 42 mm) saturated with water. Physical conditions were identical to root induction. The peat pellets were placed into small plastic greenhouses. Growing peat moss (*Sphagnum spec.*) placed in-between the peat pellets avoided a fungi attack which otherwise occurred very often.

Rooting of larch shoots is only possible if the shoots are not vitrified. Rooting rates up to 100% and high quality root systems were obtained. For optimal root development, controlled environmental conditions are very important. Slightly reduced temperature has been described as beneficial for root initiation and development (Hübl & Zoglauer, 1991). Survival of plantlets during rooting and hardening was high, if the temperature was between 17 and 18°C. In a few experiments, not reported here, an increase to 23°C had a detrimental effect on plantlet survival. The root development was undisturbed in JIFFY peat pellets and the roots received an optimal oxygen supply which was better than in agar.

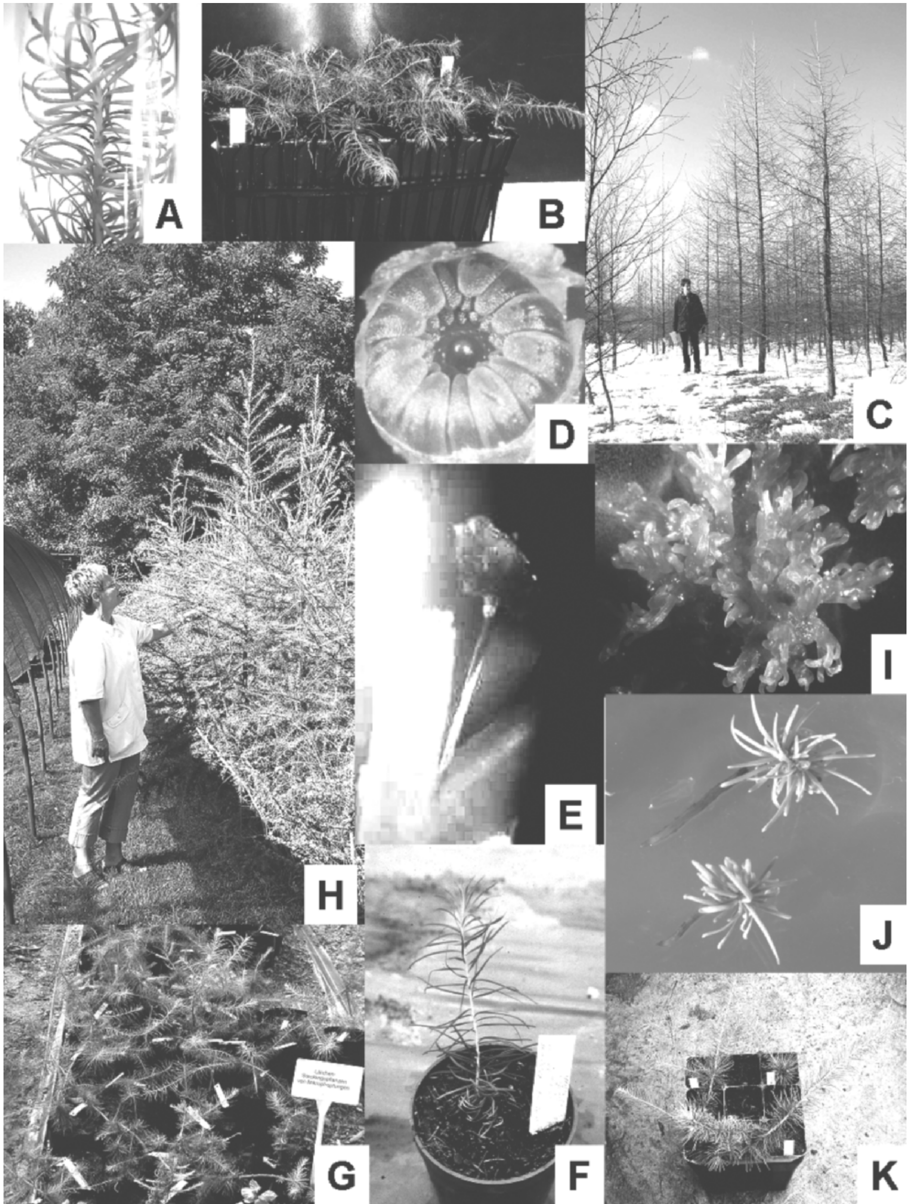

Figure 1. *Larch, Serial propagation: bud bearing long-shoot A), plagiotropic growth B), 8-year-old field trial C), Micrografting: long-shoot bud D), cut meristem E), micrografted plant F), micrograft derived cuttings G), 6-year-old cuttings H), Adventitious buds from adult plants: elongating adventious buds I), spontaneously rooted adventious buds J), elongating plants from rooted adventious buds K).*

2.4.2. Adult Material

Rooting of elongating shoots from serial propagation. Shoots derived from established propagation cultures were rooted under conditions identical to juvenile shoots, by auxin induction followed by root development in JIFFY-peat pellets.

Rooting of adventitious bud derived shoots. Although the adventitious buds had not developed into long-shoots, single adventitious buds rooted spontaneously in certain conditions. This happened within a propagation cycle during the second subculture free of phytohormones (8th to 12th week) or after additional repeated subcultures free of phytohormones (BEMB/200). It was concluded that at least a partial rejuvenation had occurred. The addition of 200 mg l^{-1} spermidine to medium BEMB/200 increased the spontaneous root formation up to 11%. Rooted adventitious buds were subcultured on medium BEMB/200 until the root reached a length of 20–30 mm.

Root development and transfer to soil. Rooted buds were transferred to a liquid medium (SH1/2) on a raft in a sterile hydroponic system. There they formed typical short-shoot characteristics. The reduced mineral medium forced root growth. Rooted short-shoots (>100 mm root length) were transferred to the soil and weaned. Some formed a long-shoot after 6–12 months and became upright growing plants (Figure 1K).

2.5. Hardening and Transfer to the Field

2.5.1. Juvenile Plant Material

After 3 months, rooted shoots were transferred together with the Jiffy-7 peat pellet into Rootrainers (RONAASH Ltd. Scotland, 4.5 × 4.5 cm, 20 cm high, each for 40 plantlets). The root was not disturbed that way and could continue growing. The containers were placed under a high-pressure-fog system until the plants resumed shoot growth. Air humidity was reduced continuously after 1 month. Plagiotropic growth behaviour became visible after 2–3 months (Figure 1B). Therefore it became necessary to transfer these plants to the nursery or field. The imbalance of fast shoot growth and slower root growth led to the appearance of plagiotropic growth (Ewald, 2000). A larch seedling is characterised by fast root growth in the first year. Based on this root, it starts orthotropic growth in the shoot. *In vitro* larch plants derived from organogenesis showed a relatively fast shoot growth. The root was formed adventitiously and therefore it had to reach an appropriate size to keep the plant growing upright (orthotropic). The faster the transfer to the field the shorter was the period until the plants grew straight. According to our observations, there was no precocious aging due to micropropagation (time until first flower formation), which might have been the case as a result of plagiotropic growth. Plants started to form the first flowers at around the age of 5–7 years like ordinary larch seedlings. The performance of juvenile larch plants propagated *in vitro* was comparable to seedlings, as shown in field trials (Figure 1C, Schneck & Ewald, 2001). Two of the best clones from a field trial were reestablished after successful testing at the age of 10 years. This showed that it was possibile to micropropagate juvenile larch, to evaluate these clones in field trials and to re-establish those with the best growth behaviour and stem form after a test period of 10 years.

2.5.2. Adult Plant Material

Plants derived from serial propagation were hardened and transferred to the field like juvenile material. Cutting-derived plants from micrografts 4 years after rooting were comparable to juvenile plants after micropropagation. This was based on all criteria observed (e.g. growth behaviour, time until flower formation). Micrografting restored the rooting capability and is, from our point of view, at present the method of choice for applying an *in vitro* method as a step in improving vegetative propagation of adult larch.

3. CONCLUSION

Larix species can be micropropagated by several methods of organogenesis using juvenile plants. Adult selected trees have been, until now, difficult to propagate due to a lack of knowledge concerning triggering of shoot elongation. Micrografting *in vitro* can support the rejuvenation process of adult donor trees to overcome difficulties in cutting propagation.

4. REFERENCES

Bonga, J.M. & von Aderkas, P. (1988) Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. In: Ahuja, M.R. & Libby, W.J. (eds.) Clonal Forestry, Vol. 1, Springer-Verlag, Berlin Heidelberg New York, pp. 182–200.

Bornman, C. (1983) Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies in vitro*. Physiol. Plant. 57, 5–16.

Boulay, M. (1979) Propagation "in vitro" du Douglas par micropropagation de germination aseptique et culture de bourgeons dormants (*Pseudotsuga menziesii* (Mirb.) Franco). Micropropagation des arbres forestiers, AFOCEL 12, 67–75.

Chalupa, V. (1991) Larch (*Larix decidua* Mill.). In Bajaj, Y.P.S. (Ed.) Biotechnology in Agriculture and Forestry 16. Trees III. Springer-Verlag, Berlin Heidelberg, pp. 446–470.

Chalupa, V. (2004) *In vitro* propagation of European larch (*Larix decidua* Mill.). Journal of Forest Science 50, 553–558.

Dembny, H. & Zoglauer, K. (1992) Bewurzelung *in vitro* etablierter Lärchensprosse. In Biotechnologische und physiologische Aspekte der pflanzlichen Zell- und Gewebekultur. Wissenschaftliche Zeitschrift der Humboldt-Universität zu Berlin 3, 107–113.

Ewald, D. (2000) Is plagiotropic growth in micropropagated larch a marker for ageing? Proceedings of the international congress "Application of biotechnology to forest genetics". Sept. 22–25, 1999 Vittoria-Gasteiz, Spanien, ISBN 84-7821-421-6, pp. 479–486.

Ewald, D. & Kretzschmar, U. (1996) The influence of micrografting *in vitro* on tissue culture behavior and vegetative propagation of old European larch trees. Plant Cell Tiss. Org. Cult. 44, 249–252.

Ewald, D. & Naujoks, G. (2000) Large scale testing of tissue culture ability of several adult larch clones and of factors influencing the growth behaviour of adult oak clones in vitro and after transfer to soil. In O'Riordain, F. (Ed.) Development of integrated systems for large-scale propagation of elite plants using in vitro techniques. COST Action 822, Report of activities 1998. ISBN 92-828-8599-2, pp. 333–335.

Ewald, D., Kretzschmar, U. & Chen, Y. (1997) Continuous micropropagation of juvenile larch from different species via adventitious bud formation. Biologia plantarum 39, 321–329.

Hübl, S. & Zoglauer, K. (1991) Entwicklung einer Vermehrungsmethode für züchterisch wertvolle Lärchen. Beitr. Forstwirtschaft 25, 18–20.

Karnosky, D.F., Huang, Y. & Shin, D.-I. (1993) Micropropagation of *Larix* species and hybrids. In Ahuja, M.R. (Ed.) Micropropagation of Woody plants. Kluwer Academic Publ., Dordrecht, pp. 373–382.

Kretzschmar, U. (1993) Improvement of larch micropropagation by induced short shoot elongation *in vitro*. Silvae Genetica 42, 163–169.

Kretzschmar, U. & Ewald, D. (1994) Vegetative propagation of 140-year-old *Larix decidua* trees by different *in-vitro*-techniques. J. Plant Physiol. 44, 627–630.
Linsmaier, E.M. & Skoog, F. (1965) Organic growth factors requirement of tobacco tissue cultures. Physiol. Plant. 18, 100–127.
Lloyd, G. & McCown, B. (1981) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Int. Plant Prop. Soc. Proc. 30, 421.
Schenk, R.U. & Hildebrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50, 199.
Schneck, V. & Ewald, D. (2001) Growth and performance of micropropagated hybrid larch clones. Silvae Genetica 50, 240–243.
von Arnold, S. & Eriksson, T. (1981) *In vitro* studies of adventitious shoot formation in *Pinus contorta.* Can. J. Bot. 59, 870–874.