ADVANCES IN BIOTECHNOLOGY OF CONIFERS

P. K. GUPTA
Biochemical Sciences Division, National Chemical Laboratory, Pune 411 008, India.

ABSTRACT

Plantlet regeneration via bud breaking, adventitious bud formation and somatic embryogenesis has been achieved from tissues, cells and protoplasts in important conifer species. The recent demonstration of genetic transformation and artificial seed production shows the potential of biotechnology in genetic improvement of conifers. The current status of biotechnology in conifer trees is reviewed.

INTRODUCTION

BIOTECHNOLOGY promises to have great impact in tree improvement (figure 1). Plant breeding has already resulted in higher-yielding plants and also resistance to some diseases and insect pests. However, in forest trees, conventional methods of selection, breeding and progeny testing are very slow and difficult. The problem is due to the long life cycle of trees (20–50 years). Conventional methods also require large spaces for screening. Regeneration from cell and tissue culture could reduce the time for selection and propagation of desirable traits. Selection of genetic variants through cell and tissue culture (somaclonal variation) requires very little space. Advances in biotechnology will rapidly accelerate tree breeding. They offer new ways to increase forest productivity and to make trees hardier and resistant to pests, diseases, herbicides and stress.

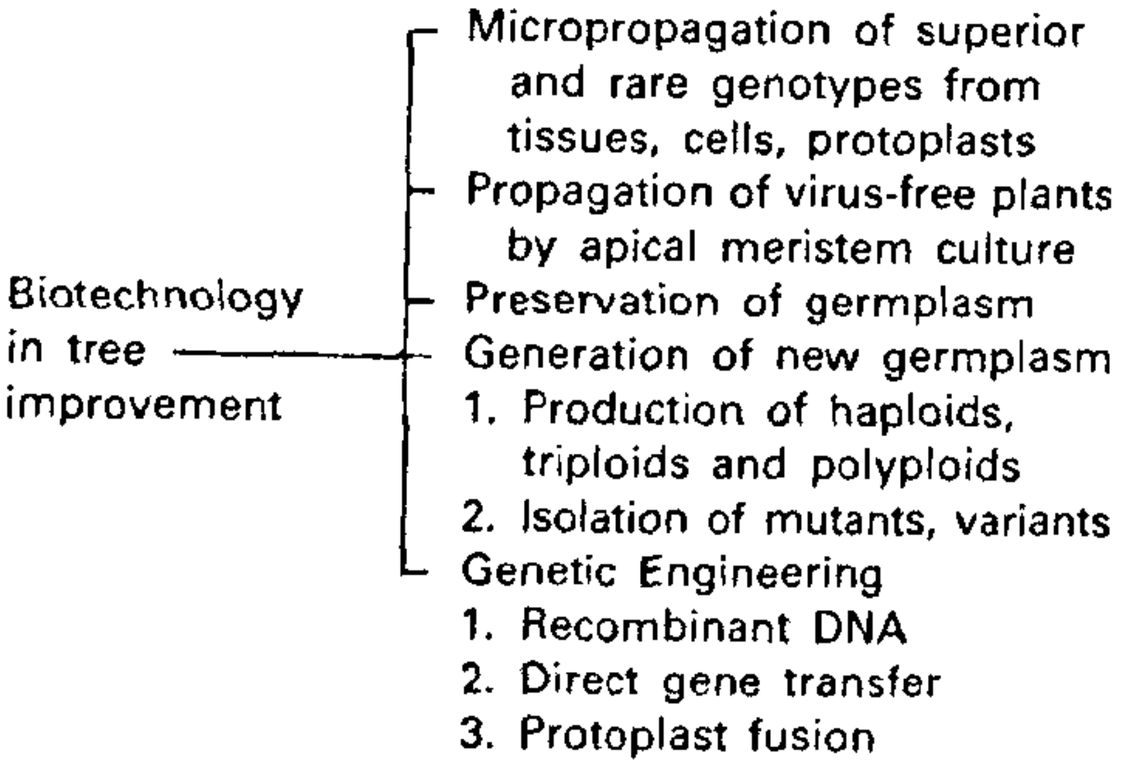

Figure 1. Tree improvement through biotechnology.

Conifers are considered as important forest species all over the world and are mostly propagated sexually through seeds. Here we briefly review the current status of *in vitro* regeneration and genetic engineering of conifer trees. Several reviews have been published on tissue culture of forest trees, which provide more detailed information and methodologies[1–8].

MICROPROPAGATION

In general, micropropagation is approached in three ways: enhancement of axillary bud break (the main method used for clonal propagation of herbaceous ornamentals, fruit trees and some hardwoods), adventitious budding (production of shoot primordia on tissue such as cotyledons, leaves, stems, etc.) and somatic or asexual embryogenesis from tissue or cells. Asexual embryogenesis is distinct from organogenesis because bipolar embryos are induced, having both a shoot and a root pole[9]. The development pattern in somatic embryogenesis is similar to that in zygotic embryogenesis.

In vitro regeneration of plantlets from juvenile explants has been achieved in a large number of economically important conifer species (table 1). However commercial propagation from juvenile tissue is still limited to 3 or 4 species: *Pinus radiata*[10] (Radiata pine) in New Zealand, and *Pinus teada*[11] (Loblolly pine) and *Pseudotsuga menziesii*[12] (Douglas-fir) in North America and France[13].

Field data for tissue culture-raised plants show uniformity in growth pattern within

Table 1 *Tissue culture of softwoods (conifers)*

Species	Explants	Mode of plantlet regeneration
Araucaria cunninghamii	Shoot tips	Budbreak
Larix decidua	Megagametophytes	Embryogenesis
Picea abies	Embryo, buds, cotyledons	Budbreak, advt. buds, organogenesis, embryogenesis
P. glauca	Cotyledons, hypocotyl, embryos	Advt. buds, embryogenesis
P. mariana	Hypocotyls	Advt. buds
P. sitchensis	Shoot tips	Advt. buds
Pinus contorta	Embryo, cotyledons, hypocotyls	Advt. buds
P. lambertiana	Embryos, cotyledons	Advt buds, embryogenesis
P. palustris	Embryos	Advt. buds
P. pinaster	Needle fascicles	Advt. buds
P. radiata	Embryos, needles, cotyledons	Advt. buds, embryogenesis
P. monticola	Embryos	Advt. buds
P. rigida	Embryos, cotyledons	Advt. buds
P. rigida × *P. taeda*	Embryos	Advt. buds
P. sylvestris	Needles, shoot tips	Budbreak, advt. buds
P. taeda	Cotyledons, embryos	Advt. buds, embryogenesis
Pseudotsuga menziesii	Cotyledons, buds (mature trees), embryos	Advt. buds, budbreak, embryogenesis
Sequoia sempervirens	Nodal segments (mature trees), shoot tips, megagametophytes	Budbreak, advt. buds, organogenesis
Thuja plicata	Cotyledons, shoot tips (mature trees)	Budbreak, advt. buds
Tsuga heterophylla	Cotyledons	Advt. buds

clones. This has been observed in all three conifer species, Douglas-fir, Loblolly pine and Radiata pine. Micropropagated Radiata pine and Loblolly pine plants showed advanced maturation characteristics. These were assumed to be due to poor root system and improper handling[11]. In contrast, Timmis[14] reported that clones of Loblolly pine propagated *in vitro* had failed to become fully cold-hardy under normally inducing conditions in the first field tests. Slowness in becoming dormant and cold-hardy is a characteristic of most juvenile plants.

Micropropagation of mature trees was not solved until recently for most of the economically important conifer species except *Sequoia sempervirens*[13]. Recently we have standardized the conditions for micropropagation of mature Douglas-fir (*Pseudotsuga menziesii*) using apical and axillary buds[15]. Season is an important factor for the tissue culture of mature trees, spring (March–April) being the best season to initiate tissue culture[16].

A new surface-sterilization method was developed by which 90% clean cultures were obtained from explants of mature trees. Shoots from bud sprouts were plagiotropic when collected from branches with horizontal and downward growth habit. In contrast, upright growth of micropropagated shoots was observed in all cases of explants from branches with an upright habit.

The newly formulated DCR medium was more effective for opening of buds, elongation, multiplication and rooting than MS and WPM media[15]. However, rooting percentage was low (10–15%). Adventitious bud formation was also achieved from needles of mature Douglas-fir after subculture[17]. Over the past three years our results have shown that micropropagation of mature Douglas-fir, though difficult, is possible and can be improved.

There are two main limitations on micropropagation of forest trees on a commercial scale. The first limitation is the uncertainty about the long-term growth behaviour of clonal material. Another limitation on clonal propagation is its higher cost. Cost studies indicate that labour inputs form a significant percentage of the total production cost[18]. The cost of planting stock

also increases sharply as *in vitro* shoot production drops, or if shoots fail to develop roots. Aitken-Christie[19] has reduced the cost of Radiata pine propagation to half by *in vitro* hedging of shoots and by addition of liquid medium to the container to avoid subculturing into another vessel. The cost of micropropagated plantlets can be further reduced by automation or (at least) by mechanization of a few stages of the process. Shoot multiplication is the stage which is most worthy of mechanization. This is because multiplication bears the highest labour costs and is the most repetitive process.

In the interim, clonal propagation can be used appropriately to establish elite trees for progeny tests and in seed orchards. Trees cloned in this way are useful for breeding and tree improvement programmes.

Somatic embryogenesis, in particular, offers tremendous potential for large-scale production of plants at lower costs. Somatic embryogenesis is the process by which somatic cells develop into differentiated plants through characteristic zygotic embryonal stages. That embryos could be regenerated from somatic plant cells in culture was first recognized by Stewart *et al*[19] and Reinert[20] using tissues from the storage tap root of the domestic carrot. Two types of somatic embryogenesis have been demonstrated: direct and indirect. Direct somatic embryogenesis refers to the development of an embryo directly from the original explant tissue. Indirect somatic embryogenesis is the formation of embryos from callus or cell suspensions. Cells which can develop into somatic embryos are said to possess embryogenic competence. The selection of specific developmental stages of an explant, conditioning media and appropriate environmental conditions, and sequential transfers, are generally necessary for successful embryogenesis. Plantlet regeneration via somatic embryogenesis has several advantages:

1. Very large numbers of plantlets can be optimally produced (e.g., 10,000/l in conifers).
2. Cell suspensions which are amenable to bioreactor technology can cut down labour, time and costs.
3. Encapsulated somatic embryos with controlled dormancy could form an efficient storage and delivery system comparable to natural seeds.
4. Presumptive rejuvenation in somatic embryos avoids maturity-related characteristics like early flowering and plagiotropic growth in plantlets.
5. Plantlets of superior quality with tap root are obtained, shoot and root meristems being an integral part of somatic embryos.
6. The cell suspension—somatic embryogenesis system is better suited for genetic engineering techniques.

In woody perennials, somatic embryogenesis has been reported in more than 25 families, 44 genera, 60 species and numerous cultivars of trees[21]. In conifers, embryoid and embryo-like structures have been reported earlier[22]. Success of somatic embryogenesis has been achieved in the last two years with Norway spruce[23–25] and *Larix*[26]. For the first time somatic polyembryogenesis (SPE) has been reported in sugarpine from 5-year-old, cold stored seeds[27]. Polyembryogenesis is common in gymnosperms, especially in conifers[28]. Polyembryogenesis is the production of more than one embryo from a single cell and SPE refers to the origin of the cell as being somatic as opposed to reproductive. Cleavage polyembryogenesis occurs when more than one embryo results by the division of the embryonal suspensor cell mass into two or more units, each developing into an embryo. Simple polyembryogenesis occurs by fertilization of several egg cells. SPE has now been extended to Loblolly pine[29] and Douglas-fir[30] which are the two most important conifer species in North America. We found that the SPE process could be used not only with callus, but also with a proliferating embryonal suspensor mass (ESM). The ESM is distinct from the callus because products of division yield not only more callus but also produce more embryonal cells indefinitely as long as the environment of the zygote can be maintained. Indeed, a

detailed analysis, confirmed in 3 other conifers, revealed that:

1. Daughter cells in the ESM repetitively produced proembryos with suspensor.
2. The predicted free-nuclear stages of an embryogenic 'basal plane' that characterizes zygotic embryogenesis could be found in cellular products of the ESM.
3. A true-to-type polyembryonic developmental process, derived from the ESM, could be traced to free nuclei by a double-staining diagnostic test specific for embryonal and suspensor cells in the ESM and in zygotic embryogenesis.
4. Nuclei in cells of the ESM have different developmental fates based on their properties. Acetocarmine-reactive products were produced by embryonal cells. Suspensor cells were permeable to Evans blue. In cell suspension cultures, the highly differentiated ESM masses often became polyembryogenic. In Douglas-fir, which does not show polyembryogenesis *in situ*, polyembryogenesis could be induced *in vitro*, e.g. on semi-solid surfaces and in suspension culture. In the suspension culture of Loblolly pine, polyembryonic structures became lignified and formed an infrastructure whereupon multiple embryos developed asynchronously.
5. The ESM retained embryogenic potential, even in mature seeds stored for up to 5 years.
6. SPE can be extended to other somatic cells of protodermal origin that contribute to the so-called somatic embryogenesis in 'callus'[23,24]. The double-staining test applied to these cells facilitates the search for other potentially embryogenic cells (ESMs) in tissues from all stages in the life cycle. The impact of SPE on mass-production forestry will be via clonal production of elite trees and enhancement of germplasm.

EXPLANT SOURCE

Explant source is an important factor in initiating the development programme of somatic embryogenesis from a cell or a group of cells. Embryonal tissues appear to be the most suitable for the induction of somatic embryogenesis. Immature embryos were found to be the most suitable for initiation of somatic embryogenesis in conifers. This has been reported for *Picea abies*[23], Douglas-fir[30], Loblolly pine[29], sugarpine[27] and *Picea glauca*[31]. Immature embryos after 4–5 weeks of fertilization were found to be the best for initiation of SPE. Somatic embryogenesis has also been initiated from mature embryos, hypocotyl and cotyledons in sugar pine and *Picea abies*.

CULTURE CONDITIONS

The following protocol is a general one that can be followed and is most suitable for Loblolly pine, Norway spruce, sugar pine, and Douglas-fir. Fine-tuning of the media and conditions may be required for different genotypes. Initially, tissues are inoculated on defined culture media every week.

MS basal medium[32] with NH_4NO_3 (550 mg/l), KNO_3 (4674 mg/l) and thiamine HCl (1 mg/l) was used. The modified MS, DCR and LP media were reduced to half strength and then supplemented with myo-inositol (1000 mg/l), sucrose (3%), L-glutamine (gln) (450 mg/l), casein hydrolysate (CH) (500 mg/l), 2,4-D (5×10^{-5} M), kinetin (Kn) and N^6-benzyladenine (BAP) (each at 2×10^{-5} M), at an initial pH of 5.7, before autoclaving. Cultures are maintained in darkness at 23 ± 2°C.

To complete early embryony the proliferating ESM bearing the early stages of SPE is subcultured on the same medium as described above except that concentration of some components was different: 2,4-D (5×10^{-6} M), Kn and BAP (each at 2×10^{-6} M). After 4 or 5 subcultures the ESM is transferred to medium containing 2,4-D (1×10^{-6} M), Kn and BAP (0.25×10^{-6} M) with ABA (1×10^{-6} M). At this stage, treatment with ABA (which inhibits polyembryogenesis but allows growth of individual embryos) is found to be beneficial for embryo development. After 3 or 4 subcultures and removal of growth regulators and ABA the globular stage of embryogenesis is fully evident. Embryos elongate and develop cotyledons within 8–10 weeks when transferred to a sterile liquid medium, with filter paper

support, without growth regulators and under continuous white light (5, 2, 0.5 $\mu W\,cm^2\,nm^{-1}$ in blue, red and far-red respectively). Complete plants are developed in a basal medium containing 0.25% w/v activated charcoal (E. Merck) and 1% sucrose. Casein hydrolysate and glutamine are removed.

For cell-suspension cultures, ESMs (ca. 2 g in 50 ml) are placed in 250 ml Erlenmeyer flasks with fluted bases and kept shaking at 120 rpm. The culture medium consists of half strength basal medium (see above) with 2,4-D (5×10^{-6} M), Kn and BAP (each 2×10^{-6} M). Cell suspensions are formed rapidly in darkness, when maintained and subcultured every 5–6 days. Repeated subculturing produces well-dispersed suspensions of single cells, aggregates of 2–5 cells and larger ESMs. The packed-cell volume is measured after centrifuging the cell suspension of each flask at 250 *g* for 10 min.

SOMATIC EMBRYO DELIVERY SYSTEM

Various delivery systems have been developed since naked embryos are in no way equivalent to a seed. It is difficult to grow somatic embryos directly in the field. Fluid drilling, encapsulation and seed tapes have been suggested as potential delivery systems[33]. Plant Genetic Inc. recently reported the successful harvest of hybrid celery and alfalfa grown from encapsulated somatic embryos[34].

Encapsulation of somatic embryos was first reported by Redenbaugh *et al*[35] for alfalfa and celery, and Kitto and Janick[36] for carrot.

In conifers (*Picea abies*, Loblolly pine and Sugar pine) the somatic embryos were mixed with 1.5% sodium alginate and dropped singly into a 50–100 mM solution of $Ca(NO_3)_2$. A coating of calcium alginate formed around the embryos and hardened to form capsules during a 20–30 min incubation. Encapsulated somatic embryos have been stored for 6 months at 4°C, without loss of viability. The development of encapsulated embryos to plants occurred on vermiculite, perlite, sand or peatplug, but at very low frequencies.

FREEZE PRESERVATION

Picea abies and *Pinus taeda* embryonal cells were frozen in liquid nitrogen (– 196°C) in a mixture of cryoprotective agents composed of polyethylene glycol, glucose and dimethylsulphoxide (10, 8 and 10% w/v). Cells were thawed rapidly and somatic embryos were regenerated from the ESMs[37]. Long-term storage in liquid nitrogen provides the opportunity to evaluate the clonal nature of plants regenerated from somatic embryos in cases where testing of progeny may take several years. The frozen cells may then be thawed, scaled-up and regenerated to achieve clonal propagation from the same embryogenic cell lines for reforestation.

PROTOPLASTS

Protoplasts are very important for genetic modification by somatic hybridization; direct gene transfer through microinjection, electroporation or via liposomes; and introduction of organelles (chloroplasts, mitochondria). Protoplast isolation followed by cell wall regeneration and cell division has been reported in several conifer species such as *Picea abies*[38], *Pinus contorta*[39], *Pinus coufteri*[40], *Pinus pinaster*[41], *Pinus sylvestris*[42], *Pinus taeda* (Loblolly pine)[43], *Pseudotsuga menziesii* (Douglas-fir)[44] and *Sequoia sempervirens*[45]. We have also reported colony formation *in vitro* by protoplasts isolated from needles of mature trees of sugar pine[46]. None of these cultures gave complete plantlet regeneration. Very recently we have obtained somatic embryogenesis from protoplasts of Loblolly pine[47] and Douglas-fir[48] using embryonal suspensor cells.

Viable protoplasts (90%) were obtained using 2–3-day-old embryonal cell suspension. Before isolation of protoplasts, embryonal cell suspensions were treated with 5000 mg/l myo-inositol for up to 3–4 subcultures (7 days intervals). Cell division occurred and microcolonies were obtained from protoplasts embedded in 0.6% agarose. Over 100 microcolonies were obtained in a regeneration medium containing myo-inositol as the major osmoticum.

Sorbitol, mannitol and sucrose were not found to be effective as osmoticum for regeneration. Proembryos were developed within 8–10 weeks from protoplast-derived colonies on solid medium. Proembryos were developed further by sequentially lowering the level of hormones and by subculturing every 15 days. Development of somatic embryos from protoplasts was observed by double staining. All stages of somatic embryogenesis were identical to stages reported earlier for somatic and zygotic polyembryogenesis. Proembryo and embryo development from protoplasts have also been achieved in Douglas-fir and White spruce[48,49] using embryonal cell suspensions.

The regeneration of somatic embryos from protoplasts opens the door for direct gene transfer and hybridization studies which were not possible earlier in conifer trees.

GENETIC ENGINEERING

Genetic engineering technology includes isolation and manipulation of genes from any living system and their transfer into bacteria, fungi, plants or animals. Genetic transformation of plants requires four steps: (i) isolation and identification of a DNA fragment consisting of a single gene or a small block of genes coding for the desired trait, (ii) insertion of the DNA into a cloning vector, where it is multiplied, (iii) transfer of the foreign DNA into the recipient cell, where it is incorporated and expressed, and (iv) regeneration of transformed cells into whole plants. Most of the successful plant transformations have been obtained using the tumour-inducing (Ti) plasmid of the soil bacterium *Agrobacterium tumefaciens* as a vector[50]. The Ti plasmid contains a piece of DNA (called T-DNA) that incorporates in the host cell genome. The T-DNA genes, expressed in transformed plant cells, include genes that are involved in the biosynthesis of opines and phytohormones (auxin and cytokinins). Another region of the Ti plasmid, the virulence (*vir*) region, codes for a function which is required for the transfer and integration of the T-DNA into the plant genome. Its normal function is to produce a tumour in the infected plant. However it can be 'disarmed' (by removal of the phytohormone-producing genes) so that the tumour does not form. The gene(s) coding for the desired trait(s) can be inserted into the T-DNA fragment, and expressed in the transformed cells.

Herbicide- and insecticide-resistant plants have been obtained through genetic engineering technology in tobacco, tomato, petunia etc[51]. Recently, genetic transformation and expression of foreign genes have been reported in forest trees such as *Populus* and Loblolly pine[52,53]. Herbicide-resistant *Populus* plants have been regenerated and are now growing on fields[54].

We have induced tumours in *in vitro* micropropagated shoots of Douglas-fir. We used two strains of *Agrobacterium tumefaciens* (K12 × 562 E and K12 × 167)[55] which contain derivatives of the wild type Ti-plasmid (pTiA6). These plasmids contain a chimeric foreign gene encoding resistance to the antibiotic kanamycin. The strains were constructed[55] by cointegrating pTiA6 and plasmid pCGN 562 (K12 × 562 E) or plasmid pCGN 167 (K12 × 167) by homologous recombination. Tumours were excised and grown on basal medium without phytohormones in the presence of 500 μg/ml carbenicillin.

OPINE DETECTION

Opines were analysed by high voltage paper electrophoresis of extracts prepared in acidified methanol[55]. Octopine was detected in all tumours obtained after infection with strains K12 × 562 E and K12 × 167 and was not present in the callus derived from untransformed cells or uninfected tissue of Douglas-fir.

KANAMYCIN PHOSPHOTRANSFERASE ACTIVITY (APH(3′)II)

Kanamycin phosphotransferase assay was performed as described earlier[56]. APH(3′)II activity was obtained in extracts from tumours obtained after infection with strains K12 × 562 E and K12 × 167. Activity was not detected in untransformed cells or tissues. The

presence of kanamycin phosphotransferase demonstrates that the foreign gene was expressed to give an active protein (APH(3')II).

SOUTHERN BLOT ANALYSIS

To confirm the stable transformation of Douglas-fir we did a Southern analysis of the genomic DNA from both transformed and untransformed cells and tissues by the method described earlier[55]. Foreign DNA (kanamycin phosphotransferase gene) was detected in all tumours and was not found in untransformed cells and in tissue of Douglas-fir.

These results show the stable transformation and expression of a foreign gene in Douglas-fir.

DIRECT GENE TRANSFER

Direct DNA transfer techniques have been developed for plant transformation to overcome the limited host range of *Agrobacterium tumefaciens*. Direct gene transfer avoids the need for introduction of cloned DNA into the T-DNA of the *Agrobacterium* Ti-plasmid before introduction into plant cells and thus also eliminates the need to remove *Agrobacterium*. It facilitates rapid analysis of gene expression. One of the most useful techniques is electroporation. Electroporation involves applying a high intensity electric field to reversibly permeabilize the cell membrane[57]. Electroporation of the cell membrane has proved useful for cell fusion as well as for gene transfer into plant and animal cells. Stable transformation and expression of foreign genes have been achieved through electroporation of plant cells[58]. Transformants have been shown to regenerate and stably pass on the introduced gene to their progeny[59].

We have also developed a transformation system using electroporation in Douglas-fir. The luciferase gene from the firefly *Photinus pyralis* was used as a 'reporter' gene. If this gene is expressed in transformed cells, its product, the enzyme luciferase, when provided with substrate, can catalyse a reaction that produces light. Gene constructs, consisting of the firefly luciferase structural gene and the 35S cauliflower mosaic virus promoter[60], were used in the electroporation experiments. The luciferase gene was successfully introduced into protoplasts of Douglas-fir and Loblolly pine by electroporation[48]. An electric pulse was delivered from a 500 μF capacitor charged to 200 V. This corresponds to an electric field intensity of 500 V/cm and RC times constant of 35. The viability of the protoplasts was reduced to 40–45% after electroporation. An extract from the electroporated protoplasts (36 and 48 h after plating) produced light when mixed with the substrates luciferin and adenosine triphosphate. Bioluminescence was measured in a Packard Picolite Luminometer. No activity was detected in extracts from protoplasts electroporated in the absence of DNA. Higher activity was detected when polyethyleneglycol was included in the electroporation buffer. Loblolly pine protoplasts gave a higher level of activity compared to protoplasts of Douglas-fir. The results clearly indicate transient luciferase gene expression in Douglas-fir and Loblolly pine protoplasts.

Earlier, coniferous trees were considered to be more difficult to regenerate than other forest trees. Now, however, somatic embryogenesis, plantlet regeneration from cells and protoplasts, and genetic transformation have opened the door to biotechnology and genetic engineering of conifer trees. The existing superior genotypes can now be exploited for the production of novel and commercially valuable genotypes.

ACKNOWLEDGEMENT

The author thanks Dr A. F. Mascarenhas, Mr E. Muralidharan and Miss Daphne Leon for the help rendered during the preparation of this manuscript.

17 March 1988

1. Karnosky, D. F., *Bioscience*, 1981, **31**, 114.
2. Mott, R. L., In: *Cloning agricultural plants via in vitro techniques*, (ed.) B. V. Conger, CRC Press, Florida, 1981, p. 217.
3. Brown, C. L. and Sommer, H. E., In: *Tissue culture in forestry*, (eds) J. M. Bonga and D. J.

Durzan, Martinus Nijhoff, The Netherlands, 1982, p. 109.
4. David, A., In: *Tissue culture in forestry*, (eds) J. M. Bonga and D. J. Durzan, Martinus Nijhoff, The Netherlands, 1982, p. 72.
5. Dunstan, D. and Thorpe, T. A., In: *5th Canadian Bioenergy R & D Seminar*, (ed.) S. Hannian, Elsevier, Amsterdam, 1984, p. 23.
6. Thorpe, T. A., *Biotechnol. Adv.*, 1983, **1**, 263.
7. Thorpe, T. A., In: *Handbook plant cell culture*, (eds) W. R. Sharp, D. A. Evans, P. V. Amirato and Y. Yamada, Macmillan, New York, 1985, Vol. 2, p. 435.
8. Thorpe, T. A. and Biondi, S. C., In: *Proc. Int. Symp., in vitro propagation forest tree spp. Azienda Regional delle Forest*, Bologna, Italy, 1985, p. 33.
9. Haccius, B., *Bot. Johrb.*, 1971, **91**, 309.
10. Aitken Christie, J., *Propagator*, 1984, **30**, 9.
11. Mott, R. L. and Amerson, H. V., In: *Proc. Symp. of recent advances in forest biotechnology, June 10–14th*, Michigan, USA, 1984, p. 24.
12. Ritchie, G. A. and D. Long, A. J., In: *The ICTCWG meeting Rotorua, 12–16th Aug.*, New Zealand, 1986, p. 80.
13. Boulay, M., In: *Annaler de Recherches Sylvicoles AFOCEL*, 1977, p. 37.
14. Timmis, R., In: *Crop physiology of forest trees*, Helsinki Univ. Press, Helsinki, 1985, p. 175.
15. Gupta, P. K. and Durzan, D. J., *Plant Cell Rep.*, 1985, **4**, 177.
16. Gupta, P. K. and Durzan, D. J., *Acta Hortic.*, 1988 (in press).
17. Gupta, P. K. and Durzan, D. J., *Am. Soc. Hortic. Sci.*, 1988 (in press).
18. Harnain, S., Pigeon, R. and Overed, R. P., *The forest chronical*, Aug. 1986, p. 240.
19. Steward, F. C., Mapes, M. O. and Mears, K., *Am. J. Bot.*, 1958, **45**, 705.
20. Reinert, J., *Planta*, 1959, **53**, 318.
21. Tulecke, W., In: *Cell and tissue culture of forestry*, (eds) J. M. Bonga and D. J. Durzan, Martinus Nijhoff, The Netherlands, 1986, p. 61.
22. Abo El-Nil, M. M., *U. S. Patent*, 1982, **353**, 184.
23. Hakman, I., Fowke, L. C., Von Arnol, S. and Eriksson, T., *Plant Sci.*, 1985, **38**, 53.
24. Krogstrup, P., *Can. J. For. Res.*, 1986, **16**, 664.
25. Gupta, P. K. and Durzan, D. J., *In vitro*, 1986, **22**, 685.
26. Nagmani, R. and Bonga, J. M., *Can. J For. Res.*, 1985, **15**, 1088.
27. Gupta, P. K. and Durzan, D. J., *Biotechnology*, 1986, **4**, 643.
28. Norstog, K., In: *Experimental embryology of vascular plants*, (ed.) B. M. John, Springer-Verlag, New York, 1982, p. 25.
29. Gupta, P. K. and Durzan, D. J., *Biotechnology*, 1987, **5**, 147.
30. Durzan, D. J. and Gupta, P. K., *Plant Sci.*, 1987, **52**, 229.
31. Lu, C. Y and Thorpe, T. A., *J. Plant Physiol.*, 1987, **128**, 297.
32. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **115**, 473.
33. Evans, D. A., Sharp, W. R. and Flick, C. E., *Hortic. Rev.*, 1981, **3**, 214.
34. Redenbaugh, K., Paasch, B. D., Nichol, J. W., Kossler, M. E., Niss, P R. and Walker, K. A., *Biotechnology*, 1986, **4**, 797.
35. Redenbaugh, K., Nichol, J., Kossler, M. E. and Paasch, B., *In vitro*, 1984, **20**, 256.
36. Kitto, S. and Janick, J. Q., *J. Am. Soc. Hortic. Sci.*, 1985, **110**, 227.
37. Gupta, P. K., Durzan, D. J. and Finkle, B. J., *Can. J. For. Res.*, 1987, **17**, 1130.
38. Chalupa, V., In: *Proc. Ist colloquium on plant tissue culture and genetic breeding*, 1973, p. 181.
39. Hakman, I. and Von Srnold, S., *Plant Cell Rep.*, 1983, **2**, 92.
40. Patel, K. R., Shekhawat, N. S., Berlyn, G. P. and Thorpe, T. A., *Plant Cell, Tissue and Organ Culture*, 1984, **3**, 85.
41. David, H., David, A. and Matille, T., *Physiol. Plant.*, 1982, **56**, 108.
42. Bornman, C. H., Personal Communication.
43. Teasdale, R. D. and Rugnini, E., *Plant Cell, Tissue, Organ Culture*, 1983, **2**, 253.
44. Kirby, E. G. and Cheng, T. Q., *Plant Sci. Lett.*, 1979, **14**, 145.
45. David, A., Personal Communication.
46. Gupta, P. K. and Durzan, D. J., *Plant Cell Rep.*, 1986, **5**, 346.
47. Gupta, P. K. and Durzan, D. J., *Biotechnology*, 1987, **5**, 710.
48. Gupta, P. K. and Durzan, D. J., *Plant Sci.*, 1988, (in press).
49. Attree, S. M., Bekkaoui, R., Dunstan, D. I. and Fowke, L. C., *Plant Cell Rep.*, 1987, **6**, 480.
50. Fraley, R. T., Rogers, S. G. and Horch, R. B., *CRC Crit. Rev.*, 1983, **4**, 1.
51. Schneiderman, H. A., *Agricultural Engineering*, 1987, p. 28.
52. Parson, T. J., Sinkar, T. J., Reinhard, F. S., Nester, E. W. and Gordon, M. P., *Biotechnology*, 1986, **4**, 533.

53. Sederoff, R., Stomp, A. M., Chilton, W. C. and Moore, L. W., *Biotechnology*, 1986, **4**, 647.
54. Fillatti, J. J., Sellmer, J. C. and McCown, B. H., *Hortic. Sci.*, 1986, **21**, 773.
55. Dandekar, A. M., Gupta, P. K., Durzan, D. J. and Knauf, V., *Biotechnology*, 1987, **5**, 710.
56. Reiss, B., Sprengel, R., Will, H. and Schaller, H., *Gene*, 1984, **30**, 211.
57. Fromm, M. E., Taylor, L. P. and Walbot, V., *Nature* (*London*), 1986, **319**, 791.
58. Fromm, M. E., Taylor, L. P. and Walbot, V., *Proc. Natl. Acad. Sci.*, 1985, **82**, 5824.
59. Shillito, R. D., Saul, M. W., Paszkowski, J., Muller, M. and Potrykus, I., *Biotechnology*, 1985, **3**, 1099.
60. Ow, D. W., Wood, K. V., DeLuca, M., Jeffrey, C., *Science*, 1986, **234**, 856.

ANNOUNCEMENTS

Dr A. P. MITRA ELECTED FOR THE ROYAL SOCIETY

It has been announced that Dr A. P. Mitra has been elected a Fellow of the Royal Society, London. He was born in 1927 and obtained the M. Sc. degree from the Calcutta University (1948) and D. Phil., Calcutta University (1955). At the invitation of the late Dr K. S. Krishnan, the then Director of the National Physical Laboratory (NPL), New Delhi, Dr Mitra joined NPL as the Secretary of the Radio Science Committee to initiate Ionospheric Data-Coordination and Prediction as a National effort. In 1957 he was nominated the Secretary of the National Committee for the International Geophysical Year (IGY). The coordinated national efforts under a system of controlled intercomparison and specified conditions were, perhaps, introduced in India for the first time and Mitra played a critical role in this with guidance from Krishnan. He was also the Secretary of the National Committee for IQSY and the Indian programme's implementation continued to be his responsibility. By this time the Radio Propagation Unit had been formed at NPL with Mitra as its Head. A series of investigations in the area of space research were undertaken.

The period between 1965 and 1980 was one of intense activity on radio patrol of solar flares (with techniques covering a wide range of frequencies: VLF, LF, MF, HF, VHF and microwaves) on a wide variety of rocket payloads (riometer receiver, gerdien condensers, propagation payloads, etc.); the special satellite radio beacon campaign during ATS-6 operation; multi-frequency riometer observations, etc. Ion chemistry work emerged as a major activity.

In 1970's he was asked to serve as a member of the Task Force to plan and organize research efforts leading to large scale troposcatter systems in the country.

Between 1980 and 1982, Dr Mitra worked as a Jawaharlal Nehru Fellow on changing environment. He took over the Directorship of the National Physical Laboratory (NPL) in April 1982. In February 1986, he was appointed the Director-General of the Council of Scientific and Industrial Research and the Secretary to Govt. of India, Department of Scientific and Industrial Research.

Dr Mitra has played a vital role in the development of ionospheric research in India. As Secretary of the Indian National Committee he guided the IGY and the IQSY programmes in India; introduced radio communication predictions that now cover frequencies from a few kilohertz to many gigahertz and serve all radio traffic organizations in India; established the Associate Regional Warning Centre (ARWC) that helps in rapid dissemination of solar and geophysical data over the Indian subcontinent; guided the development of ionospheric research in many of the Indian universities and institutions abroad; has produced students who now lead ionospheric activities in India and abroad; has been intimately involved with many International bodies (especially COSPAR, SCOSTEP and URSI).

Dr Mitra is the author of 16 books and monographs and over 140 research papers and reviews. His book on Ionospheric Effects on Solar Flares was published (Reidel Publishing Co., Holland) in 1974 and its Russian Translation appeared in 1977. The Handbook on Radio Propagation for Tropical and Subtropical countries, edited by Dr A. P. Mitra and colleagues was highly acclaimed as a remarkable achievement at the meeting of the International Union of Radio Science held at Tel Aviv in 1987.

Dr A. P. Mitra is a Fellow of the National Institute of Science of India (Now Indian National Science Acadamy— 1961); corresponding Member (1963) of the International Academy of Astronautics