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Thin Cell Layers: Application to Forestry Biotechnology

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ABSTRACT

Thin cell layers or TCLs have been one of the most recent yet important techniques for the tissue culture and micropropagation of select forestry species. In this review, we abridge the current state of application of TCL technology to forest tree biotechnology, focusing on *Pinus* spp., *Paulownia* spp. and *Populus* spp. Where conventional methods have not always produced ideal results in the micropropagation of forestry species, TCL technology has now perfected the ability to control developmental and morphogenetic processes *in vitro* at a finer scale than the use of conventional explants focusing the size and the origin of the explant to ensure successful regeneration for micropropagation and transformation studies. TCLs are fundamental in implementing lab-based experiments to larger scale field-based applications and its application and proven success with three select forestry and timber species provides a frame-work for the development of the technique for other forestry species.

Keywords: callogenesis, caulogenesis, organogenesis, rhizogenesis, somatic embryogenesis

CONTENTS

INTRODUCTION

Thin cell layers (TCLs) is a term that was coined by Kim Tran Thanh Van in 1973, exactly 35 years ago in two key publications (Torrigiani *et al*. 1973; Tran Thanh Van 1973). Even though the concept of totipotentiality (totipotency), introduced by Haberlandt had already existed for 75 years, and even through the sterile culture of plant cells and organs had already been well established by 1973, TCLs transformed plant tissue culture by being able to control more strictly the outcome of an organogenic "programme", primarily by controlling the size of the explant. Despite the concept of totipotency (Konar and Nataraja 1965; Nataraja and Konar 1970), woody forest trees remained difficult-to-propagate species.

TCLs of plant tissues from dozens of plant species covering many plant families have been used successfully as explants for *in vitro* plant regeneration in many plant species (Mulin and Tran Thanh Van 1989; Tran Thanh Van and Van Le 2000; Nhut *et al.* 2003a; Nhut *et al.* 2003b; Teixeira da Silva 2003; Malabadi and van Staden 2003; Malabadi *et al*. 2004a; Malabadi *et al.* 2004b; Teixeira da Silva 2005; Malabadi *et al.* 2005; Malabadi and Nataraja 2007; Teixeira da Silva 2008, 2010; Teixeira da Silva and Tanaka 2010). TCL technology considers cells and tissues as the most fundamental developmental building blocks (Teixeira da Silva *et al*. 2007). The TCL system allows a specific cell or tissue layer to be isolated, and, depending on the genetic state and epigenetic requirements, in conjunction with strictly controlled growth conditions (light, temperature, pH, PGRs, media additives, among others) may lead to the *in vitro* induction of morphogenic programs (Teixeira da Silva *et al*. 2007). The capacity of a TCL to enter a program depends upon a number of factors, including correct signal perception and transduction, the capacity of the internal genetic machinery to respond and react to these signals and in the latter case, may depend on the physiological state and origin (tissue and organ) of the TCL, environmental stress, chemical stress factors applied to the TCL (Teixeira da Silva *et al.* 2007).

TCL technology is usually more refined than conventional explant preparation since it requires an extremely focused eye and a very stable hand. Thus this method should not, where possible, be used as the first line of attach to an experimental hypothesis. Rather, where the desired organogenesis cannot be achieved or where a species is apparently recalcitrant to regeneration *in vitro*, then TCL technology may prove useful. This would be the case for woody plants, specifically forest tree species, the focus of this mini-review.

CELL DIVISION IN TISSUE AND ORGAN FORMATION

Higher plants develop from a single-celled zygote into a multicellular organism through co-ordinated cell divisions, and when this process occurs without patterning, disorganized callus tissue is produced (Teixeira da Silva and Nhut 2003a). Body organization is generated by two distinct processes: the first, the primary organization of the body, rep-

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Invited Mini-Review

resented by the seedling, and including the shoot and root meristems, is laid down by embryonic pattern formation, while the meristems then take over to produce the adult plant during post-embryonic development. Plant organs are composed of ordered collections of various cell types differing in their shape, size, position, function and DNA content. Cell division, separation and morphogenesis are interlinked, and ontogenesis is determined by the genome and is influenced by external signals. Correct control of the cell-division cycle is required for the elaboration and execution of developmental programmes, while patterning genes determine overall architecture of the plant. Cell division, a critical activity during the growth and development of a plant, together with cell signalling (Wu *et al.* 2002), provides the building blocks for the differentiation of *in vitro* TCLs or *in planta* tissues and organs, and contributes to the overall size of the individual.

This review serves to show readers the application of the concept of the TCL model to forestry species. There are not many cases, and most have been achieved successfully by the first authors and colleagues over the past ~5 years. But the examples that exist are crystal-clear and serve as powerful guidelines for the application of TCL technology to other forestry species where the technique has not yet been applied, e.g. *Salix* spp., *Eucalyptus* spp., Sandal wood (*Santalum album*), teak (*Tectona grandis*), *Dalbergia sisso*, *Cryptomeria japonica*, *Pinus densiflorum*, and many pines and spruce species of different geographical locations. TCL technology is also a solution to many of the issues currently hindering the efficient progress of forest tree improvement, since it resolves problems at the first stage, i.e. regeneration by using the most basic developmental building blocks, cells and tissues. Through the use of TCLs the regeneration of specific organs may be effectively manipulated and, together with specific controlled *in vitro* conditions and exogenously applied plant growth regulators (PGRs), many problems hindering the improvement of *in vitro* plant systems are potentially removed, as has been reviewed by Nhut *et al*. (2006). The possibility of TCLs as a developmental tool for molecular and genetic studies is put into perspective. The TCL system also allows for the mass propagation of several forestry species of economic interest and has thus profound potential financial benefits and positive applications to biodiversity conservation, mass propagation for reforestry, secondary metabolite production through bioreactor technology using somatic embryos derived from TCLs. The TCL system could in theory provide a simple but efficient micropropagation system for developing countries with limited resources and facilities while it could be used as a high-tech method for the production of perfect, uniform clonal forestry species in an ever-increasingly demanding market with increasingly rigorous environmental restrictions.

Readers should note that the initial concept of a TCL was applied to thin sections of *N. tabacum* pedicels (Tran Thanh Van 1973). One mm-thick layer of cells with variable area dimensions were defined as a longitudinal TCL or lTCL, while a transverse slice, a few mm thick, was termed a transverse TCL or tTCL. In a recent paper, the second author contested this terminology originally used and now widely adapted, and suggest that the term be adjusted to Thin Tissue Layer or TTL (Teixeira da Silva 2008).

Paulownia

The early works (Burger *et al*. 1985; Rao *et al*. 1996; Bergmann and Moon 1997) on the *in vitro* propagation of paulownia inspired the subsequent studies using TCLs (Nhut *et al*. 2003e). Following the removal of leaves, *Paulownia fortunei* tTCLs (1 mm) from young stem segments of 1 year-old trees were used to initiate cultures. Stem segments (5-10 mm in diameter, I cm long) containing one node were disinfected for 20 sec in 70% ethanol, then immediately soaked in 0.6% sodium hypochlorite (i.e. active chlorine concentration) solution for 15 min followed by several thorough rinses in sterile distilled water (SDW). tTCLs were prepared from these stem segments, placed on basal MS medium with 100 mg/l *myo*-inositol, 0.5 mg/l nicotinic acid, o.5 mg/l pyridixine-HCL, 1 mg/l thiamine and 2 mg/l glycine. Optimal shoot induction from tTCLs occurred on this basal medium supplemented with 30 g/l sucrose, 7 g/l agar and 5 µM BAP, forming an average of 8 shoot buds per tTCL and with 87% of tTCLs forming shoots. Double the optimal concentration of BAP, or none whatsoever resulted in no shoot formation. However, when 0.1 µM NAA was added to 5 µM BAP, the number of shoot buds per tTCL increased to 12, with a 92% response rate. Callogenesis could also be strictly controlled by adding, instead of 0.1 µM NAA, 5-10 µM. *In vitro* rhizogenesis from TCLs (or from any explant for that matter) has not been reported, nor has somatic embryogenesis.

Pines

Successful initiation of embryogenic tissue derived from vegetative shoot apices of mature trees (14-20 years old) using TCL technology has been reported for the first time in many recalcitrant pines for example, *Pinus kesiya* (Malabadi *et al*. 2004), *P. roxburghii* (Malabadi 2006; Malabadi and Nataraja 2006a, 2006b), *P. wallichiana* (Malabadi and Nataraja 2007), *P. patula* (Malabadi and van Staden 2003; Malabadi and van Staden 2005a, 2005b, 2005c; Malabadi and van Staden 2006), and *P. sylvestris* (Aronen *et al.* 2007, 2008), *P. contorta* (Lodgepole pine) (Park *et al.* 2009), *P. pinea* (Portuguese stone pine) and *P. pinaster* (Portuguese Maritime pine) (Malabadi *et al*. unpublished work). Induction of somatic embryogenesis using TCL of apical shoots and secondary needles of mature trees has been well established in *P. roxburghii* (Malabadi and Nataraja 2006, 2007). Embryogenic tissue derived from the cloning of mature pines using TCL technology serves as the best starting material for genetic transformation studies in conifers. The first report of genetic transformation using biolistic method including the isolation of cDNA clones of genes has was reported using TCL technology-induced-embryogenic-tissue in *P. roxburghii* (Malabadi and Nataraja 2007a, 2007b). The first transgenic trees were produced in an Indian pine *P. roxburghii* following biolistic gene transfer (Malabadi and Nataraja 2007d), and *Agrobacterium*-mediated genetic transformation using embryogenic tissue of mature trees of *P. roxburghii* (Malabadi *et al*. 2008), followed by the *Agrobacterium*-mediated genetic transformation of embryogenic tissue of mature Himalayan blue pine (*P. wallichiana*) (Malabadi and Nataraja 2007e). The homeobox transcription factor *WUSCHEL* (*WUS*) has been shown to cause de-differentiation when expressed on somatic cells that can lead to somatic embryogenesis or organogenesis (Zuo *et al*. 2002). The expression of *WUS* gene in the embryogenic tissue derived from the mature trees of *P. roxburghii* revealed that *WUS* might be influencing the molecular mechanism that mediates the vegetative-to-embryogenic transition (Malabadi and co-workers, unpublished work). The WUS gene was isolated and identified from the embryogenic tissue during cloning mature trees of *P. roxburghii*. This gene was not expressed in the non-embryogenic tissue and therefore, WUS might be involved in the conversion of somatic cells into embryogenic pathway. This was the first report of involvement of *WUS* gene during cloning mature trees of *P. roxburhii* (Malabadi and coworkers, unpublished work). This is the major breakthrough in forest biotechnology and might help in solving the current problems of transformation of recalcitrant pines, and TCL technology has many potential applications in commercial forestry.

Khasi pine (*P. kesiya* Royle ex. Gord), Chir pine (*P. roxburghii* Sarg), and Himalayan blue or Bhutan pine (*P. wallichiana* AB Jacks) are three commercially important Indian pines. *P. kesiya* is an economically important early successional species which is predominant in the subtropics (800-2000 m above sea level) of North East India extending from East Khasi Hills of Meghalaya state up to Myanmar and Philippines (Malabadi *et al.* 2004) whereas Chir pine (*P.*

roxburghii) distributed throughout all parts of India (Malabadi and Nataraja 2006). Himalayan blue pine or Bhutan pine is a native of the outer Himalaya and prevalent in the Northern Himalayan range. It is an important indigenous pine species in India, Bhutan and Nepal. Very few trees of *P. wallichiana* were also available in the Western Ghat Forests as a result of shifting cultivation trial program by forestry department for the conservation of important forestry species. Ecology and economy of Indian region and of its people is greatly influenced by these three pine species to a larger extent. These pines not only provides timber, fuel wood and pulpwood, but also meets the demand for packing cases, stakes for vegetable cultivation, bedding for cattle sheds, and cushion material for packaging of fruits and vegetables. Resin obtained from these pines is a product of great industrial importance as it is used in soap, paper and pharmaceutical and paint industries (Malabadi and Nataraja 2006). Embryogenic cultures were first time established using TCL in *P. kesiya*. Precultured TCLs of apical shoot buds of mature trees of *P. kesiya* (15 years old) on DCR basal medium containing 0.3% of activated charcoal for 3 days produced enormous mass of embryogenic tissue on DCR induction medium supplemented with 22.62 µM 2,4-D, 26.85 µM NAA and 8.87 µM BA (Malabadi *et al.* 2004). Partial desiccation of embryogenic tissue for 24 h prior to transfer to maturation DCR basal medium containing 37.84 µM and 5 g l^{-1} Gellan gum stimulated maturation of somatic embryos in *P. kesiya* (Malabadi *et al*. 2004). In the case of *P. roxburghii* (Chir pine), TCLs of apical shoot buds precultured at 4°C for 3 days in the dark also produced embryogenic tissue when subcultured on DCR-induction basal medium supplemented with 22.62 µM 2,4-D, 26.85 µM NAA and 8.87 µM BA. Maturation frequency was also very high (61.8%) after 24 h of desiccation treatment prior to maturation (Malabadi 2006; Malabadi and Nataraja 2006). Precultured TCL of secondary needles from mature (14-year-old) trees of *P. roxburghii* at 4°C for 3 days in the dark produced embryogenic tissue on DCR basal medium supplemented with 22.62 µM 2,4-D, 26.85 µM NAA and 5 µM triacontanol (Malabadi and Nataraja 2007b). In *P. wallichiana* (Himalayan blue or Bhutan pine), TCL of apical shoot buds were able to produce embryogenic tissue on DCR basal induction medium, after the addition of 10% smoke saturated water derived from the local grasses in DCR basal medium (Malabadi and Natarja 2007a). This was achieved by slow burning of a mixture of two local semi-dry grasses *Aristida setacea* and *Cymbopogon martini* (Graminiaceae) (Malabadi and Nataraja 2007a). These observations suggest that active ingredient (s) in smoke saturated water play a regulatory role plant embryogenesis. In case of *P. patula*, cold-pretreatment of TCL of apical shoot buds at 2°C for 3 days on 0.3% activated charcoal induced white mucilaginous embryogenic tissue on DCR-induction-medium supplemented with 20 µM 2,4-D, 25 µM NAA and 9 µM BA (Malabadi and van Staden 2003, 2005a, 2005b, 2005c, 2006). Partial desiccation of embryogenic tissue for 24 h prior to transfer to maturation medium containing 9 $g1^{-1}$, 80 µM ABA enhanced somatic embryo maturation and germinability (Malabadi and van Staden 2003, 2005a, 2005b, 2005c). Storability and germination of sodium alginate encapsulated somatic embryos derived from TCL of vegetative shoot apices of mature *P. patula* trees were tested on DCR basal medium (Malabadi and van Staden 2005a, 2005b, 2005c). This study for the first time reports somatic embryos produced by TCLs of apical shoot buds encapsulated with 2.5% sodium alginate dissolved in DCR basal salts gave significantly higher germination (89%) than other treatments. Germinated synthetic seeds produced normal plantlets in *P. patula* (Malabadi and van Staden 2005c). Culture of TCL of apical meristem is one of the frequently used strategies for commercial micropropagation of plants. This technique enables the mother plants genetic features to be conserved maximally in the regenerated plants (Malabadi and van Staden 2003, 2005a, 2005b, 2005c, 2006). Therefore, TCL technology has great potential for application in commercial forestry (Malabadi and van Staden 2005a, 2005b, 2005c). In case of *P. sylvestris*, TCLs of vegetative shoot apices also produced enormous mass of embryogenic tissue on DCR induction medium supplemented with 20 µM 2,4-D, 25 µM NAA and 9 µM BA (Aronen *et al.* 2007). This also holds good for other recalcitrant pines such as *P. pinea* and *P. pinaster* where, TCL of vegetative shoot buds influenced the induction of embryogenic tissue on DCR basal medium (Malabadi *et al*. unpublished work).

Of the various alternatives for cloning elite conifers, somatic embryogenesis (SE) appears to be the best option. In recent years, significant areas of lodgepole pine (*Pinus contorta*) forest have been devastated by the mountain pine beetle (MPB) in Western Canada. Recently, Park *et al*. (2009) established a SE propagation system for MPB resistant lodgepole pine using TCL layers, several families displaying varying levels of resistance were selected for experimentation involving shoot bud and immature seed explants. In bud cultures, eight embryogenic lines were induced from two of 15 genotypes following various treatments. Genotype had an important influence on embryogenic culture initiation, and this effect was consistent over time. These embryogenic lines were identified by microscopic observations and genetic markers. Further putative embryogenesis-specific genes, *WOX2* and *HAP3a*, were analyzed in cultures of both shoot bud explants and ZE. Based on these analyses, it was postulated that *WOX2* and *HAP3a* could be used as early genetic markers to discriminate embryogenic cultures from callus. This study provides interesting information that will be used in future research on propagation of mature pines (Park *et al.* 2009).

Hence the above studies confirmed that TCL technology has a tremendous potential for application in commercial forestry. TCL technology is very simple and cost effective too. A deeper understanding of earlier, non-TCL studies on regeneration of Monterrey pine were covered by Nhut *et al*. (2003e) and serve as an excellent basis for comparison with TCL systems developed by Malabadi and colleagues. Early studies by the Thorpe/Yeung group (Villalobos *et al*. 1995) also provide a fundamental understanding of TCL systems to this economically important conifer species.

Poplar

Lee-Stadelmann *et al*. (1989) compared the effect of explant size on shoot regeneration of hybrid Populus NE 299 (*P. nigra* var. *betulifolia* x *P. trichocarpa*). Young, but fully expanded leaves at the third node from the top were removed from containerized, greenhouse-grown trees. Leaves were surface sterilized in 1% sodium hypochlorite with a few drops of Tween 20 for 7 min, followed by three rinses with SDW. Specifically the capacity of "micro-cross sections" (syn. tTCLs) of 100, 200, 300, 400 or 500 µm made through leaf mid-veins vs. 400 m sections of different widths (<1 mm, 1-2 mm, or >3 mm) to produce shoot buds was compared. These sections were cut using a vibrating microtome from the leaf beginning, i.e. from about 1 cm distal to the petiole attachment since this zone was already known by the same group to form adventitious shoots. Woody plant medium containing 2% sucrose, 0.2 mg/l BA and 0.01 mg/l NAA was optimal. tTCLs formed 25 times more shoots than conventional, larger (1 cm) explants; in both cases, explant orientation was not critical.

Approximately 90% of the tTCLs formed green callus, the remainder non-chlorophyllous callus: only green callus could form shoots.

PERSPECTIVES

When one considers the success with which almost half a dozen pine species have been tissue cultured using TCL technology there is the sense that the possibility of using this simple but efficient system for a much wider range of conifers and other forestry species. The direct application of

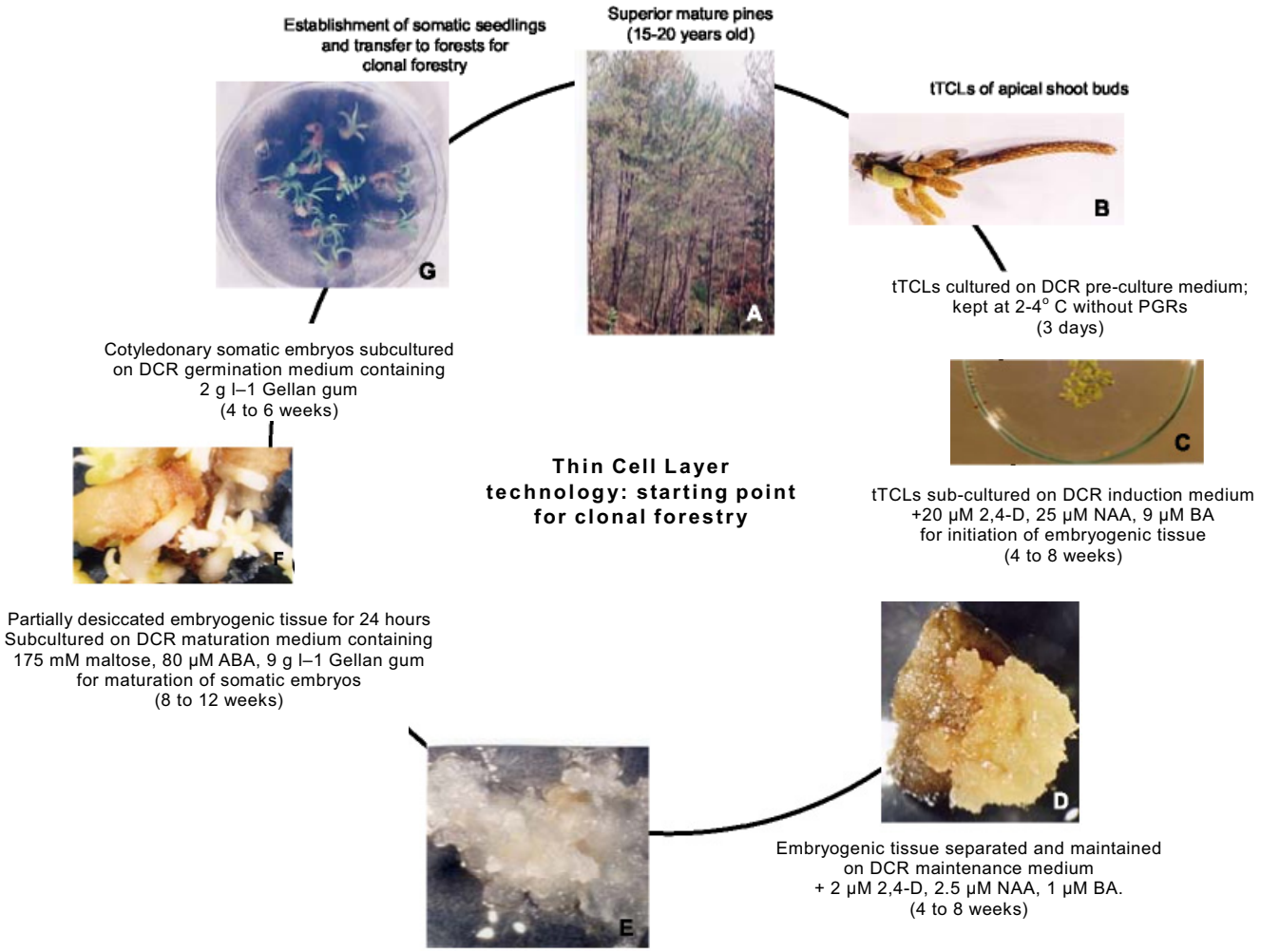

Fig. 1 The application of TCL technology in clonal forestry.

TCL technology to plant genetic engineering (Malabadi and Natarja 2007e, 2007f; Malabadi *et al.* 2008), as a tool to study *in vitro* flowering (Teixeira da Silva and Nhut 2003b) or plant physiology. Use of TCL technology for the induction embryogenic system has been reported in many pine species and an embryogenic system could be used for genetic transformation studies (Malabadi and Nataraja 2007e, 2007f; Malabadi *et al.* 2008). Another important advantage of using TCL of vegetative shoot apices of mature pines as a starting material for genetic transformation is that cells are actively dividing, hence their higher regeneration capacity, and serve as the best starting material for biolistic transformation. These cells are generated by the meiotic division of meristematic tissue, and meristematic cells possess higher regeneration potential, withstand higher biolistic pressure showing maximum cell integrity compared to cells derived from embryo cloning (Malabadi and Nataraja 2007d). Another reason might be that during cloning of mature trees, the single somatic cell of TCL explant is programmed towards embryogenesis under the stress conditions of cold-pretreatment (Malabadi *et al*. 2004, 2009; Malabadi and van Staden 2003, 2005a, 2005b, 2005c, 2006; Malabadi 2006). Stress induced by cold-pretreatment might make the cells more resistant, and are ready to withstand biolistic pressure resulting in the compact cell integrity of cells (Malabadi and Nataraja 2007d). On the other hand the cells resulting from embryo cloning are much elongated and loosely arranged cells since they are originated not due to any stress conditions but from the embryo only that resulted in the bursting and loss in cell integrity during biolistic transformation (Malabadi and Nataraja 2007d). This might help in solving the current problems of regeneration of transgenic lines by biolistics using TCL technology. This will also result in the stable transformation of a particular tree line under study, and the transgenic lines could be used for commercial forestry since they have defined genetic characters of superior parents.

Recently transgenic trees produced by using embryogenic tissue derived from cloning mature trees using TCL technology by biolistic-mediated transformation were reported in an Indian pine *Pinus roxburghii* (Malabadi and Nataraja 2007d). The transformation efficiency was higher than our other studies of *P. kesiya* and *P. wallichiana* (Malabadi and Nataraja 2007g, 2007h) by using the embryogenic tissue derived from TCL of mature trees, and also resulted in the stable expression of transgenes (Malabadi and Nataraja 2007d). In another study, the embryogenic tissue of TCL explants of mature trees of *P. wallichiana* was also successfully used for genetic transformation studies, and resulted in the production of transgenic plants in three lines using *Agrobacterium*-mediated genetic transformation (Malabadi and Nataraja 2007e). Therefore, the transgenic lines are clearly defined with genetically-inherited characteristics of their parents. This is the main advantage of cloning mature trees, and could be used for the biolistic-gene-transformation in the remaining conifers. Therefore, it is also concluded that the starting explant material particularly TCL (**Fig. 1**) might also play an important role in genetic transformation, and a good tissue culture protocol is very much needed for the successful regeneration of plantlets from transgenic tissue. The establishment of a method for the efficient regeneration of one particular species is crucial for its transformation. This is the most important factor for solving the current problems of genetic transformation studies, particularly the regeneration of transgenic plants of woody plant species with TCL technology.

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