Research in Biotechnology, 2(6): 01-07, 2011 ISSN: 2229-791X
www.researchinbiotechnology.com

Review Article

Journey of a single cell to a plantlet *via in vitro* cloning mature trees of conifers

Ravindra B. Malabadi[1]*, K. Nataraja[1], S. Vijaya Kumar[2], Gangadhar S. Mulgund[1]

[1]Department of Botany, Karnatak University, Pavate nagar, Dharwad-580003, Karnataka, India
[2]Department of Biotechnology, Madanapalle Institute of Technology and Science, Madanapalle-517325, Chitoor District, Andhra Pradesh, India
*Corresponding author Email: mlbd712@rediffmail.com

During cloning of mature conifers, isolated somatic cells from apical meristematic tissue under any external stress conidions of cold\heat or chemical are induced to form a somatic embryo. This review paper highlights the difference between embryogenesis patterns in angiosperms and gymnosperms and updates information on the current progress made in the cloning of mature trees of conifers. Insights gained through these systems has already lead to the development of cloning methodologies that could aid in reprogramming apical meristematic cells of recalcitrant mature conifers for clonal forestry.

Key words: Cloning, India, meristematic cells, mature pines, somatic embryogenesis, Western Ghat forests

Plant cells are totipotent. Somatic embryogenesis is the best example and evidence of totipotency, and is used as a model system for studying the mechanisms of de-defferentiation and re-differentiation of plant cells (Feher *et al.* 2003; Ikeda-Iwai *et al.* 2003; Mordhorst *et al.* 1997; Namasivayam, 2007; Toonen *et al*. 1994). It is still unclear how a external applied stress conditions such as cold\heat or chemical stimuli changes a somatic cell has to undergo in order to become an embryogenic cell and capable of forming an embryo at a later stages of development (Feher *et al.* 2003;Namasivayam, 2007). In general, an embryoid may arise from a single cell, or a group of cells, budding, depending on neighbour relationship of cells within the explant (Williams and Maheswaran, 1986; Feher *et al.* 2003; Namasivayam, 2007). In plants, cell division continues in specialized meristem regions such as those at the apices of primary roots and stems. As these regions are displaced distally by the cells they create, they leave behind cells that cease division but continue in growth and therefore, expand extensively (Zimmerman, 1993; John and Qi, 2008). The embryogenic cells are very important because they differentiate, and undergo cleavage polyembryony to form somatic embryos at a later time in conifers. This review paper highlights the difference between embryogenesis patterns in angiosperms and gymnosperms and updates information on the current progress made in the cloning of mature trees of conifers.

[1]Present address: Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, 11304-89 Avenue, Edmonton, T6G 2N8, Canada

Plant embryogenesis; Angiosperm versus Gymnosperm

Zygotic embryogenesis is the result of the fertilized egg cell. In flowering plants, sexual reproduction involves double fertilization that gives rise to an embryo and the suspensor simultaneously. Meiosis precedes the formation of gametes and fertilization restores the somatic chromosome number. Conifer embryos arise from a single fertilization event within the ovule, creating a diploid embryo that develops within a haploid female gametophyte (Konar, 1963; Choudhury, 1962; Dogra, 1967; Singh, 1978; Nagmani *et al.* 1995). In the majority of angiosperms, the first division of the zygote is asymmetric and gives rise to a small apical cell and a large basal cell. The fates of the apical and basal cells are clearly distinct, resulting in the formation of octant stage as a two tires towards the formation of embryo proper by apical cell. The basal cell forms suspensor and the very basal end of the embryo in *Arabidopsis* (Jurgens, 2003). In angiosperms, the endosperm (triploid tissues arising as a result of double fertilization) may surround the developing embryo and supplies the nutrients to the developing embryo e.g. *Arabidopsis.* Endosperm may be absorbed during the development in the common bean (*Phaseolus vulgaris*). In contrast, in case of gymnosperms, the nucleus in the zygote divides so that four free nuclei are formed, which become arranged in a tier (Choudhury, 1962; Konar, 1963; Singh, 1978). After several divisions, the proembryo becomes cellularised. Conifer embryos develop within the female gametophyte; no endosperm is present in conifer seeds (Choudhury, 1962; Konar, 1963; Singh, 1978). However, the gymnosperm embryos are surrounded by the megagametophyte (haploid maternal tissue). Gymnosperm zygote undergoes several rounds of nuclear divisions without cytokenesis to enter a free nuclear phase after fertilization which is followed by cellularization to form two tiers to form four tiers. Cells of the first and second tiers will multiply to form the embryo proper, while cells of the third and fourth tiers will elongate but undergo limited cell division to form the embryonal suspensor (Choudhury, 1962; Konar, 1963; Dogra, 1967; Singh, 1978; Nagmani *et al*. 1995). The outer layer of cells in embryonal mass divides periclinally, but also anticlinally, thereby not permitting the differentiation of the classical protoderm (Konar, 1963; Singh 1978). Another important step during plant embryogenesis is the establishment of the plant axis. First, the apical root meristem is formed. Later, the shoot apical meristem and cotyledon primordial are organized at the distal part of the embryo proper. Once both meristems are delineated, the plant axis becomes established. Multiple embryos are found commonly within the early-stage seeds of conifers. These multiple embryos may be formed via two processes. In simple embryony egg cells within different archegonia are fertilized by different pollen grains, resulting in zygotes of different genotype within the seed. A process called cleavage polyembryony wherein the immature embryos are multiplied. The fertilized embryos within the seed then divide into four embryos (cleavage polyembryony), and thus up to 16 embryos may form within each seed in *P. roxburghii* (Konar, 1963; Singh, 1978). Of the 10 genera in the family *Pinaceae*, only *Cedrus*, *Pinus*, *Tsuga*, *Keteleeria* were reported to show cleavage polyembryony (Konar, 1963; Dogra, 1967). Some species of Douglas-fir (*Pseudotsuga menziesii*) do not show cleavage polyembryony during somatic embryogenesis (Hong *et al.* 1991). In conifers, out of two embryos, one embryo within the seed becomes dominant by unknown processes, and continues to grow and develop. The subordinate embryo(s) do not develop but persist briefly in the ovule and appear to be the initiating material for somatic embryogenesis in some pines/ or

ultimately degraded, by programmed cell death (PCD) (Filonova *et al*. 2002). In both gymnosperm and angiosperms, seeds are designed to supply the embryo with nutrients and signaling molecules, as well as to protect the embryo from different stresses and premature germination. The mature seeds are classified as orthodox or recalcitrant (Engelmann, 1991). The embryos of orthodox seeds undergo maturation drying while recalcitrant seeds do not and are generally desiccation intolerant. The majority of angiosperm and gymnosperm seeds are of the orthodox type. At the end of the maturation phase, seeds of the orthodox type enter dormancy, including that physiological processes stop and the water content rapidly decreases (Goldberg *et al*. 1989).

Cloning mature trees of conifers

Embryo cloning was well established in conifers, and somatic embryogenesis was first reported in *Picea abies* (L.) Karst. (Hakman and von Arnold, 1985), *Larix deciduas* Mill (Nagmani and Bonga, 1985), and in *Picea abies* (Gupta and Durzan, 1986). Somatic embryogenesis has since been initiated in other conifers, including several pine species. Embryo cloning system is the most common method of somatic embryogenesis in many conifers since it is easily applicable to many pine species. The most common explant in conifer somatic embryogenesis for cloning is immature zygotic embryos. During cloning, fertilized megagametophytes from seeds are excised and placed on an appropriate medium to permit the extrusion of embryogenic tissue from the micropylar end. The problem with this method is numerous genetically undefined somatic embryos often form in the extruded material which can then be subcultured to a multiplication medium (Gupta and Durzan, 1985). However, use of an embryo as an explant has several disadvantages including heterozygosity as a result of cross-pollination (Malabadi and van Staden, 2005a, 2005b, 2005c). Immature zygotic embryos (actually whole megagametophytes containing multiple zygotic embryos) are induced to undergo what might best be described as continuous cleavage polyembryony following extrusion of the zygotic embryos from the megagametophyte (Becwar *et al.* 1991). Thus, while the zygotic embryos from which the cultures are initiated may represent superior half-sib or even full-sib families (if they are the product of controlled pollinations), the fact remains that they are unproven genetically. To add to the uncertainty of the genetic value of material propagated via somatic embryogenesis, many workers have observed that embryogenic cultures are usually not initiated from the dominant zygotic embryo in the megagametophyte, but rather from one of the subordinate embryos that would most likely have aborted had the seed been allowed to mature (Becwar *et al*. 1991). Furthermore, it was shown that a certain percentage of the embryogenic cultures initiated using this approach may actually be mixtures of genotypes, derived from multiple zygotic embryos that were present in the megagametophyte at the time of extrusion (Becwar *et al.* 1991). Another major drawback of embryo cloning is very low initiation frequencies of embryogenic tissue which is less than 2 to 3% in most of the conifers. Along with low initiation frequencies, tissue maintenance particularly cryopreser-vation, and processing is very expensive due to multi step tissue culture procedures. This limits the embryo cloning and deployment of plants for the clonal forestry. Even if there is any success of embryo cloning, the deployment of somatic seedlings in the field trial is a waste process for the clonal forestry due to undefined genetic material, and for the assessment of genetic characters, it may take another 15-25 years as a period of time for the breeder for the assessment of genetic characters in the offspring. These drawbacks of the current

approach for initiating embryogenic pine cultures from seed embryos could be avoided if a method was available for initiating embryogenic cultures from tissues of mature, proven pine trees. However, mature tree tissues of most pines are known to be highly recalcitrant to vegetative propagation of any kind and the general consensus is that they must be "rejuvenated" to make them amenable to propagation *via* such approaches as rooted cuttings or tissue culture, including somatic embryogenesis.

At present an embryogenic system derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers (Bonga and Pond, 1991; Ruaud *et al.* 1992; Bonga and von Aderkas, 1993; Ruaud, 1993; Westcott, 1994; Litz *et al*. 1995; Smith, 1994, 1997; Paques and Bercetche, 1998; Bonga, 1996, 1997, 2004; Malabadi *et al.* 2004; Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007, 2008; Malabadi and Nataraja, 2007a, 2007f, 2007g, 2007e; Malabadi *et al.* 2008a, 2008b; Park *et al.* 2009;Malabadi *et al.* 2009; Malabadi and Teixeira da Silva, 2011). Another important advantage of using vegetative shoot apices of mature pines as a starting material for somatic embryogenesis is that cells are actively dividing, hence their higher regeneration capacity, and serve as the best starting material for genetic transformation studies. These cells are generated by the active division of meristematic tissue, and meristimatic cells possess higher regeneration potential, withstand higher biolistic pressure showing maximum cell integrity compared to cells derived from embryo cloning (Malabadi and Nataraja, 2007a). Another reason might be that during cloning of mature trees, the single somatic cell is programmed towards embryogenesis under the stress conditions of cold-pretreatment (Malabadi *et al*. 2004; Malabadi and van Staden, 2005a, 2005b, 2005c). 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, 2007b, 2007c). Recently transgenic trees produced by using embryogenic tissue derived from cloning mature trees by biolistic-mediated transformation were reported in *Pinus roxburghii* (Malabadi and Nataraja, 2007a). The transformation efficiency was higher than our other studies of *P. kesiya* and *P. wallichiana* (Malabadi and Nataraja, 2007b, 2007c) by using the embryogenic tissue of mature trees, and also resulted in the stable expression of transgenes (Malabadi and Nataraja, 2007a). In another study, the embryogenic tissue 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).

Conclusion

The cloning of mature conifers using apical meristematic tissue is one of the novel approaches for the clonal forestry. There are many differences between embryogenesis patterns in angiosperms and gymnosperms. The the use of stress conditions either cold or heat or chemical treatment of apical meristematic cells in combination with other culture conditions, has the potential to induce somatic embryogenesis in recalcitrant conifers.

References

Aronen TS, Pehkonen T, Malabadi RB, Ryynanen L (2008) Somatic embryo-genesis of Scots pine-advances in pine tissue culture at Metla. Vegetative propagation of conifers for enhancing land scaping and tree breeding. Proceedings of the Nordic meeting held

in September 10th-11th 2008 at Punkaharju, Finland. *Working Papers of the Finnish Forest Research Institute,* **114**, 68-71.

Aronen TS, Ryynanen L, Malabadi RB (2007) Somatic embryogenesis of Scots pine: initiation of cultures from mature tree explants and enhancement of culture system [Abstract]. In: *IUFRO Tree Biotechnology Conference*, June 3-8, 2007, Ponta Delgada, Azores, Portugal, No. SIX. 2.

Becwar MR, Blush TD, Brown DW, Chesick EE (1991) Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. *Plant Cell Tissue Organ Culture*, **26**: 37-44.

Bonga JM, Pond SE (1991) Adventitious shoot formation in cultures of 30-year old *Larix deciduas*, *L. leptolepis*, and *L. laricina* trees. *Plant Cell, Tissue and Organ Culture*, **26**, 45-51.

Bonga JM, von Aderkas P (1993) Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. In: Ahuja MR, Libby WJ (Eds) *Clonal Forestry I, Genetics and Biotechnology*, Springer-Verlag, Berlin, 1993, pp 182-199.

Bonga JM (1996) Frozen storage stimulates the formation of embryo-like structures and elongating shoots in explants from mature *Larix deciduas* and *L.* × *eurolepos*. *Plant Cell, Tissue and Organ Culture*, **51**, 195-200.

Bonga JM (1997) The effect of collection dates and frozen storage on the formation of embryo-like structures and elongating shoots from explants from mature *Larix deciduas* and *L. x eurolepis*. *Plant Cell, Tissue and Organ Culture*, **51**, 195-200.

Bonga JM (2004) The effect of various culture media on the formation of embryo-like structures in cultures derived from explants taken from mature *Larix deciduas*. *Plant Cell, Tissue Organ Culture*, **77**, 43-48.

Choudhury CR (1962) The embryogeny of conifers: a review. *Phytomorphology,* **12**:313-338.

Dogra PD (1967) Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in *Pinaceae*. Stud Forest Suec **45**:5-97.

Engelmann F (1991) *In vitro* conservation of tropical plant germplasm: a review. *Euphytica*, **57**:227-243.

Feher A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell Tissue Organ Culture,* **74**: 201-228.

Filonova LH, von Arnold S, Daniel G, Bozhkov PV (2002) Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. *Cell Death Diff*, **9**:1057-1062.

Goldberg RB, Barker SJ, Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. *Cell*, **56**: 149-160.

Gupta PK, Durzan DJ (1986) Somatic poly-embryogenesis from callus of mature sugar pine embryos. *Bio/Technol*, **4**:643-645.

Hakman I, von Arnold S (1985) Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). *J Plant Physiol*, **121**:149-158.

Hong L, Boulay M, Gupta PK, Durzan DJ (1991) Variations in somatic polyembryogenesis: induction of adventitious embryonal-suspensor masses on developing Douglas fir embryos. In: Ahuja MR, ed. Woody Plant Biotechnology, New York, NY, USA: Plenum, 105-121.

Ikeda-Iwai M, Umehara M, Satoh S, Kamada H (2003) Stress-induced somatic embryogenesis in vegetative tissues of

Arabidopsis thaliana. *Plant* Journal, **34**:107-114.

John PCL, Qi R (2008) Cell division and endoreduplication: doubtful engines of vegetative growth. *Trend Plant Science*, 13 (3):121-126.

Jurgens G (2003) Growing up green: cellular basis of plant development. Mech Deve **120**: 1395-1406.

Konar RN (1963) Anatomical studies on Indian pines with special reference to *Pinus roxburghii* Sarg. *Phytomorphology,* **13**:388-402.

Litz RE, Moon PA, Chavez VM (1995) Somatic embryogenesis from leaf callus derived from mature trees of the cycad *Ceratozamia hildae* (Gymnospermae). *Plant Cell, Tissue and Organ Culture*, **40**, 25-31

Malabadi RB, van Staden J (2003) Somatic embryos can be induced from shoot apical domes of mature *Pinus patula* trees. *South African Journal of Botany*, **69**, 450-451.

Malabadi RB, Choudhury H, Tandon P (2004) Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. *Scientia Horticulture*, **102**, 449-459.

Malabadi RB, van Staden J (2005a) Somatic embryogenesis from vegetative shoot apices of mature trees of *Pinus patula*. *Tree Physiology*, **25**, 11-16.

Malabadi RB, van Staden J (2005b) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. *In Vitro Cellular and Developmental Biology– Plant*. **41**, 181-186.

Malabadi RB, van Staden J (2005c) Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell Tissue and Organ Culture*, **82**, 259-265.

Malabadi RB, van Staden J (2006) Cold-enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium. *South African Journal of Botany*, **72**, 613-618.

Malabadi RB (2006) Effect of glutathione on maturation of somatic embryos derived from vegetative shoot apices of mature trees of *Pinus roxburghii*. *Journal of Phytological Research*, **19**, 35-38.

Malabadi RB, Nataraja K (2006a) Cryopreservation and plant regeneration *via* somatic embryogenesis using shoot apical domes of mature *Pinus roxburghii* Sarg. trees. *In Vitro Cellular and Developmental Biology – Plant*, **42**, 152-159.

Malabadi RB, Nataraja K (2006b) RAPD detect no somaclonal variation in cryopreserved cultures of *Pinus roxburghii* SARG. *Propagation of Ornamental Plants*, **6**, 114-120.

Malabadi RB, Nataraja K (2007a) Smoke-saturated water influences somatic embryogenesis using vegetative shoot apices of mature trees of *Pinus wallichiana* A. B. Jacks. *Journal of Plant Sciences*, **2**, 45-53.

Malabadi RB, Nataraja K (2007e) Genetic transformation of conifers: Applications in and Impacts on commercially forestry. *Transgenic Plant Journal*, **1**(2), 289-313.

Malabadi RB, Nataraja K (2007f) Plant regeneration *via* somatic embryogenesis using secondary needles of mature trees of *Pinus roxburghii* Sarg. *International Journal of Botany*, **3**, 40-47.

Malabadi RB, Nataraja K (2007g) Isolation of cDNA clones of genes differentially expressed during somatic embryogenesis of *P. roxburghii*. *American Journal of Plant Physiology*, **2**, 333-343.

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008a) A new approach involving salicyclic acid and thin cell layers for cloning mature trees of *Pinus roxburghii*

(Chir Pine). *The American Journal of Plant Science and Biotechnology*, **2**(2), 56-59.

Malabadi RB, Teixeira da Silva JA, Nataraja K (2008b) Salicylic acid induces somatic embryogenesis from mature trees of *Pinus roxburghii* (Chir pine) using TCL technology. *Tree Forestry Science and Biotechnology*, **2**(1), 34-39.

Malabadi RB, Mulgund GS, Vijay Kumar S (2009) How somatic cells follows embryogenic pathway during cloning mature trees of conifers? *Journal of Phytological Research*, **22**(1), 53-56.

Malabadi RB, Teixeira da Silva JA (2011) Thin cell layers: Application to forestry biotechnology. *Tree and Forestry Science and Biotechnology*, **5**(1), 14-18.

Mordhorst AP, Toonen MAJ, Vries SC (1997) Plant embryogenesis. *Critical Review Plant Science,* **16**:535-576.

Nagmani R, Bonga JM (1985) Embryogenesis in subcultured callus of *Larix deciduas*. *Canadian Journal of Forest Research*, **23**:873-876.

Nagmani R, Diner AM, Garton S, Zipf AE (1995) Anatomical comparison of somatic and zygotic embryogeny in conifers. In: Jain S, Gupta P, Newton R, eds. Somatic embryogenesis in woody plants, vol 1. Dordrecht, the Netherlands: Kluwer Academic, 23-48.

Namasivayam P (2007) Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell Tissue Organ Culture*, **90**:1-8.

Park SY, Klimaszewska KK, Malabadi RB, Mansfield SD (2009) Embryogenic cultures of lodgepole pine originating from mature trees and from immature seed explants. IUFRO Tree Biotechnology Conference, June 28th-July 2nd 2009, Whistler, BC, Canada, p 60 (abstract).

Paques M, Bercetche J (1998) Method for rejuvenating gymnosperms by somatic embryogenesis. Patent no. PCTWO9923874A1, Paris.

Ruaud JN, Bercetche J, Paques M (1992) First evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. *Plant Cell Reports*, **11**: 563-566.

Ruaud JN (1993) Maturation and conversion into plantlets of somatic embryos derived from needles and cotyledons of 7-56-day-old *Picea abies*. *Plant Science*, **92**: 213-220.

Singh H (1978) Embryology of gymnosperms. In: *Handbuch der Pflanzenanatomie* (Encyclopedia of Plant Anatomy), vol. 10, Part 2. 1978 Berlin, Germany: Gebruder Borntraeger.

Smith DR (1997) The role of *in vitro* methods in pine plantation establishment: The lesson from New Zealand. *Plant Tissue Culture Biotechnology*, **3**: 63-73.

Smith DR (1994) Growth medium for plant embryogenic tissue. Australia/Canada Patent #PM5232.

Toonen MAJ, Hendriks T, Schmidt EDL, Verhoeven HA, van Kammen A, deVries SC (1994) Description of somatic-embryo forming single cells in carrot suspension cultures employing video cell tracking. *Planta*, **194**:565-572.

Westcott RJ (1994) Production of embryogenic callus from nonembryonic explants of Norway spruce *Picea abies* (L.) Karst. *Plant Cell Reports*, **14**: 47-49.

Williams EG, Maheshwaran G (1986) Somatic embryogenesis: factors influencing coordinate behavior of cells as an embryogenic group. *Annals of Bototany*, **57**:443-462.

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. *Plant Cell*, **5**: 1411–1423.