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(REVIEW ARTICLE)

WUCHEL Gene Family: Transcription Factor-*WOX2* As A Early Genetic Marker of Gene Expression During Induction of Somatic Embryogenesis: An updated Review

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Abstract

WUSCHEL-related homeobox (*WOX*) is a large group of transcription factors (*WOX1-WOX14*) specifically found in plants. *WUSCHEL* homeobox 2 (*WOX2*) is important for regulating many aspects of plant somatic embryogenesis. Transcription factors (TFs) are master regulators involved in controlling different cellular and biological functions as well as diverse signaling pathways in plant growth and development. The transcription factors like *WUSCHEL*-RELATED HOMEOBOX (*WOX2*) are crucial for maintaining cellular totipotency and regulating the developmental pathways of somatic embryogenesis. The induction of somatic embryogenesis using shoot apical thin layers has been successful by Malabadi and co-workers in few conifers such as *Pinus roxburghii*, *Pinus kesiya*, *Pinus wallichina*, *Pinus patula,* and *Pinus sylvestrus* (Scots pine). Furthermore, the detection and expression of *PrWOX2*, and *PcWOX2* in embryogenic cultures imitated from shoot tip thin layers of mature trees of *P. roxburghii* and Lodgepole pine (*Pinus contorta*) could be used as a genetic marker for the identification of embryogenic tissue in pines. The findings from this study based on the molecular assessment, suggested that the cell lines derived from bud cultures were truly embryogenic, not just cells that imitate embryogenic cultures (EC) in morphology. Moreover, these experimental observations also suggest that *Pr WOX2* and *PcWOX2* could be used as an early genetic marker to discriminate embryogenic cultures from callus initiated from thin cell layers of mature trees of conifers. The research contribution of pine tissue culture work by Indian plant biotechnology Stalwarts, Professor RN Konar, Nagamani, P Mahshwari, Professor Pramod Tandon, Professor Chittaranjan R. Deb, Professor Hiranjit Choudhary, Dr. Sarita Arya, Dr. ID Arya, Dr. RK Kalia, and Dr. Rajani S. Nadagouda has been updated and discussed. **The Government of India** has awarded **Professor Pramod Tandon** the fourth highest civilian honour **Padma Sri in 2009**, for his outstanding contribution to plant science. However, commercialization of pine tissue culture work in India is still facing problems and major bottleneck due to many reasons. Applications of cell sorting techniques, embryogenic cell culture identification by Artificial Intelligence (AI) should be applied for the future studies of initiatation of embryogenic cultures using thin cell layers of shoot apical domes of mature conifers.

Keywords: Conifers; Indian Pine; Reprogramming; Somatic Embryogenesis; *WOX2*; *WUCHEL*

1. Introduction

The number of *WOX* genes in the plant body increases as plants grow more complex and varies in different species. WUSCHEL-related homeobox (*WOX*) is a large group of transcription factors specifically found in plants [1-35]. *WOX* members contain the conserved homeodomain essential for plant development by regulating cell division and differentiation [1-45]. Transcription factors (TFs) regulate several cellular and metabolic activities, as well as signalling pathways in plants during stress, growth and development. The WUSCHEL-RELATED HOMEOBOX (*WOX*) genes are

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transcription factors (TFs) that are part of the homeodomain (HD) family, which is important for the maintenance of apical meristem, stem cell niche, and other cellular processes [1-25]. Transcription factors (TFs) are crucial for the transcriptional and post-transcriptional control of genes involved in response to environment stress response [1]. *WOX* gene family play an important role in the whole plant's growth and development, such as in the stem, embryo, root, flower, and leaf [1-45]. Many studies using *Arabidopsis* as a model plant have increased our knowledge of the function of embryogenesis-related genes in angiosperm [1-35]. Numerous research studies have discovered that the *WOX* gene family play a role in the whole plant's growth and development, such as in the stem, embryo, root, flower, and leaf [1-50]. Transcription factors (TFs) are crucial for the transcriptional and post-transcriptional control of genes involved in response to environment stress response [1-20]. *WOX2* increases the expression of the auxin transporter *PIN1* gene to regulate stem cell organogenesis in *Arabidopsis* [4]. *PpWOX2* (*Pinus pinaster*) over-expression enhanced somatic embryogenesis and plant organ formation in *Arabidopsis* transgenic seedlings [5].

Linkage analysis of *WOX* protein sequences demonstrated that amino acid residues 141–145 and 153–160 located in the homeodomain are possibly associated with the function of *WOXs* during the evolution [1-75]. These 350 members were grouped into 3 clades: The first clade represents the conservative *WOXs* from the lower plant algae to higher plants; The second clade has the members from vascular plant species; The third clade has the members only from spermatophyte species [1-65]. Furthermore, among the members of *Arabidopsis thaliana* and *Oryza sativa*, the ubiquitous expression of genes in the first clade and the diversified expression pattern of *WOX* genes in distinct organs in the second clade and the third clade has been observed [1-75-79].

Sarkar et al., (2007) [44] reported that throughout the lifespan of a plant, which in some cases can last more than one thousand years, the stem cell niches in the root and shoot apical meristems provide cells for the formation of complete root and shoot systems, respectively [44]. Both niches are superficially different and it has remained unclear whether common regulatory mechanisms exist [44]. In the root niche the quiescent centre cells, surrounded by the stem cells, express the homeobox gene *WOX5* (WUSCHEL-RELATED HOMEOBOX 5), a homologue of the WUSCHEL (*WUS*) gene that non-cell-autonomously maintains stem cells in the shoot meristem [44]. Loss of *WOX5* function in the root meristem stem cell niche causes terminal differentiation in distal stem cells and, redundantly with other regulators, also provokes differentiation of the proximal meristem [44]. Sarkar et al., (2007) [44] also indicated that conversely, gain of *WOX5* function blocks differentiation of distal stem cell descendents that normally differentiated [44]. Importantly, both *WOX5* and *WUS* maintain stem cells in either a root or shoot context [44]. Sarkar et al., (2007) reported that stem cell maintenance signalling in both meristems employs related regulators [44]

Rasheed et al., (2024) [1] reported that the *WOX* transcription factors (TFs) play a significant role in plant growth and development and biotic and abiotic stresses responses [1]. For example, it was found that *WOX2* increases the expression of the auxin transporter *PIN1* gene to regulate stem cell organogenesis in *Arabidopsis* [4]. **Malabadi et al., (2011)** has reported the expression of *PrWOX2* gene during induction of somatic embryogenesis from apical buds of mature trees of *P. roxburghii* [3-5]. Malabadi et al., (2011) confirmed that ***PrWOX2*** gene was used as the marker of embryoigenic cultures initiated from mature tree buds of *Pinus roxburghii* [3-5]. *PpWOX2* (*Pinus pinaster*) overexpression enhanced somatic embryogenesis and plant organ formation in *Arabidopsis* transgenic seedlings [6]. To detect the expression of the WUSCHEL (*WUS*) gene during somatic embryogenesis in plants, one can utilize techniques like quantitative real-time PCR (qRT-PCR) to measure mRNA levels or in situ hybridization to visualize *WUS* expression within specific tissues [1-29]. Additionally, reporter gene assays, such as using a *WUS* promoter-*GUS* fusion, can help to track *WUS* expression patterns during somatic embryo development. Transcription factors are an extensive group of regulatory proteins that play significant roles in different aspects such as growth and development by connecting their target genes through particular binding domains and controlling their expression [1-67]. Previous study has shown that the *WOX* transcription factor gene family has a function in the structuring of several early plant cell populations [1-20-45]. Components of the *WOX* protein family are crucial for the upkeep and growth of stem cells in the cambium, the lateral meristem that gives rise to all of the cellular parts of the wood [1-20]. Prior research has indicated that *WOX* genes are important for the advancement and progress of plants [1-25-44].

2. An Overview of WOX Gene Family

The *WUSCHEL* (*WUS*) gene in plants was discovered by Thomas Laux, Klaus F. X. Mayer, Jürgen Berger, and Gerd Jürgens [1-70]. They identified it as essential for maintaining the integrity of shoot and floral meristems in *Arabidopsis* [1-2-75]. Their work, published in 1996, established *WUS* as a key player in stem cell regulation in plants. *WOX* gene family was first discovered in *Arabidopsis thaliana* in 1996, with its essential role in shoot and floral development [1, 7]. The term "HOMOBOX" refers to a gene that can cause a single component of the cell to undergo metamorphosis when it develops from one embryo stage to another which was first proposed by William Bateson [1, 8].

The *WOX* transcription factors were classified into 14 subfamilies, which include *WOX* (homeobox related to WUSCHEL), *BELL* (homeodomain similar to BELL), *PINTOX, NDX* (homeobox NODULIN), *KNOX* (homeobox KNOTTED like), *LD* (luminidependens homeodomain), *PHD* (plant homeodomain with a finger domain), *HD-ZIP I–IV* (homeodomain leucine zipper), *ZF-HD* (zinc finger home- odomain), and *DDT* (homeodomain *DDT*) [1, 10-55]. High-affinity monomers of HDs attach to DNA by interactions with the helix–turn–helix (HTH) structure [1, 9]. The *WOX* gene family is divided into **three Clades**: Ancient, Intermediate, and modern (*WUS*) based on historical evolution linkage [1, 11]. Among 15 *WOX* genes in *Arabidopsis*, the protein of the modern clade is the largest which contains *WUS, WOX1/6, WOX2, WOX3, WOX4,* and *WOX5/7* subclades and, in the modern/*WUS* clade, *WOX* genes also contain conserved domains such as the ERF-associated amphiphilic repression (EAR) domain and *WUS* motifs other than the homeodomain [1, 11]. The word "*Wuschel*" refers to the bristling and branched phenotype of altered plants, where ectopic meristems repeatedly form and degenerate before they mature [1, 7, 9, 10]. Apart from the highly conserved homeodomain region, there is minimal sequence similarity across *WOX* proteins in the various subfamilies [39]. In several plant domains, *WOX* roles have diversified to provide unique features that regulate cell identification [1, 7, 9, 10]. The *WOX* family was identified in various plants, sunflower, wheat, black cottonwood, European spruce maize, sorghum, rice, arabidopsis, peach, tea plant, coffee plant, sweet orange, chinese plum, pine, Chinese red pine, maiden-hair tree, cotton, blueberry, and walnut [1, 7, 9, 10-20-75-79].

WOX1 works as a controller and detector that takes part in transportation of auxin polarity in leaf-limited area and detects the restriction caused by auxin transduction [1-25]. *WUSCHEL* homeobox 2 (*WOX2*) is important for regulating many aspects of plant somatic embryogenesis [1, 3-6, 16-19, 26, 27, 29]. *WOX2* over-expression in *Arabidopsis* enhanced organogenesis and somatic embryogenesis in a portion of the first- and second-generation transgenic seedlings [1-25]. Kadri et al., (2021) [29] reported that *WUSCHEL* over-expression promotes callogenesis and somatic embryogenesis in *Medicago truncatula* Gaertn [29]. Previous research has shown that *WOX3* genes are essential for the lateral organs' development of their lateral domains [1-25]. *WOX4* is a key regulator of cell identity and division activity in the vascular cambium of hybrid aspen [1-25]. In adult *Pinus sylvestris*, *PsWOX4*'s maximum transcript level occurred during the cambial zone's active cell proliferation phase [1, 15]. This tree also had the highest cambial age, 63 years, which was associated with the cambial zone's highest number of cell layers [1-25]. The root apical meristem (RAM) and the shoot apical meristem (SAM) of higher plants are where stem cell niches are most apparent [1, 30-33]. The *WOX5* gene encodes a transcription factor, which is an essential regulator, preserving the composition and functionality of the stem cell niche in plant root tips [1, 30-33]. *WOX5* is essential for maintaining the stem cell niche in the root apical meristem [74]. In contrast, *WOX5* inappropriately suppresses genes associated with shoot formation, likely via inhibiting shoot growth [1, 30-33]. Ectopic expression of *WOX5* in *Arabidopsis thaliana* probably represses shoot-related genes, which lead to inhibition of shoot development [1, 30-33]. *WOX6/PFS2* controls ovule development and influences ovule patterning [1, 33-38]. The low expression of *WOX7* resulting from low plant sugar concentration and the promoting roles of sugar and other nutrients, such as nitrogen and phosphate, prevent *WOX7*'s inhibitory effect on lateral root development under ideal growth conditions [1, 33-38]. *WOX8* and *WOX9* are expressed in the zygote throughout development and in the basal offspring following zygotic division [1, 33-38]. Both genes are necessary for embryo and shoot development [1,33-38]. It was reported that over expression of the *CsWOX9* gene in cucumber regulates wart formation in cucumber fruit [1, 38].

WOX11 has a role in the establishment of unexpected roots (also called adventitious roots) in both *Arabidopsis* and rice [1, 39-40]. *WOX11* overexpression can strengthen the capacity of falling leaves to develop roots, whereas *WOX11* suppression can result in a reduction in root formation [1, 39-40]. *Arabidopsis WOX11* is essential for starting new organs by growing secondary roots from several leaves, rebuilding adventitious lateral roots from wounded primary roots, and forming calluses in tissue culture [1, 39-40]. *WOX11* promotes overall plant growth and development by inhibiting nematode-induced limitation of primary root growth [1, 39-40]. Plants develop more secondary roots to modify their root structure in reaction to nematode infection [1]. When the main root is broken, auxin response sites in the promoter region of *WOX11* produce a local buildup of auxin, activating the protein's transcriptional activity [1-38]. The HOMEOBOX13 (*WOX13*) gene in *Arabidopsis* controls fruit patterning by suppressing the expression of the JAG/FIL genes in the medial domain which, in turn, permits proper replum formation [1-40]. *WOX13* plays a crucial role in regulating callus development and organ reconnection [1, 41-43]. Research demonstrates that when tissue is injured in the leaf petiole and hypocotyl of *Arabidopsis*, *WOX13* is transcriptionally activated [1, 41-43]. *WOX14* functions in the cambial zone to promote cell differentiation as compared to cell proliferation [1, 41-43]. This is in opposition to prior research that hypothesized *WOX14* and *WOX4* work in coordination to promote cell proliferation [1, 41-43]. The *WOX* genes work in regulating other genes to activate their function. *WUS* expression initiates in the embryo at the 16-cell stage in the apical cells of domains and during post-embryonic development in the central part of the floral meristem and in the inflorescence meristem, as well as in the expressing cells of the organizing center [1, 41-43]. Rasheed et al., (2024) [1] reported *WUS* regulates the expression of genes that are involved in auxin synthesis and response, including

TIR1, TAR2/, MP/ARF5, and *TMO6* [1, 41-43]. *WUS* also maintains the level of auxin response in the shoot apical meristem at a stable and low level, but not at zero [1-44].

Transcription factors (TFs) are master regulators involved in controlling different cellular and biological functions as well as diverse signaling pathways in plant growth and development [1, 2, 7- 39-40]. WUSCHEL (*WUS*) is a homeodomain transcription factor necessary for the maintenance of the stem cell niche in the shoot apical meristem, the differentiation of lateral primordia, plant cell totipotency and other diverse cellular processes [1- 7- 39-40]. Recent research about *WUS* has uncovered several unique features including the complex signalling pathways that further improve the understanding of vital network for meristem biology and crop productivity [1, 2, 7- 39-40]. In addition, several reports bridge the gap between *WUS* expression and plant signaling pathway by identifying different *WUS* and WUS-related homeobox (*WOX*) genes during the formation of shoot (apical and axillary) meristems, vegetative-to-embryo transition, genetic transformation, and other aspects of plant growth and development [1- 7- 39-40-75]. In this respect, the *WOX* family of TFs comprises multiple members involved in diverse signaling pathways, but how these pathways are regulated remains to be elucidated [1, 2, 7- 39-40-65].

According to Rasheed et al., (2024) [1] the *WOX* gene family plays an important role in regulating plant growth and development and response to environmental stresses [1-40]. The role of *WOX* genes in abiotic stress tolerance, such as to drought, salt, and cold, highlights their potential for biotechnological applications in crop resilience augmentation [1-50]. However, significant problems still exist in transferring insights from model species to economically relevant crops. Future studies should focus on extending the functional genomics of *WOX* genes in non-model plants and investigating their interaction networks with other transcription factors [1-50]. Using techniques like *CRISPR*-based gene editing to investigate *WOX* gene activities across multiple plant species would be useful in developing crops with greater resistance to environmental challenges, hence aiding sustainable agriculture [1-50].

Rasheed et al., (2024) [1] also indicated that the study of *WOX* genes is promising for future prospects, but it still faces various challenges [1-50]. The *WOX* gene family shows a wide diversity across plant species, contributing to plants' development and abiotic stress response [1-44]. However, limited genomic data make it difficult to implement these findings in agricultural applications, especially in non-model plants [1-50]. The complex interactions of *WOX* genes with other transcription factors (e.g., *CLAVATA*) are also not well understood, which may lead to varied effects depending on the species [1-67].

WOX genes comprise a large plant-specific gene family that belongs to the homeodomain (HD) class of transcription factors [1-60]. *WUS* HDs are critical for early-phase embryogenesis and lateral organ development [1-76]. HD proteins typically contain a highly conserved motif of 60 amino acids encoded by a characteristic DNA fragment called the homeobox and consisting of a helix-loop-helix-turn-helix structure [1-76]. In one of the studies reported by Zhao et al., (2015) [76], the WUSCHEL (*WUS*)-related homeobox (*WOX*) gene family coordinates transcription during the early phases of embryogenesis [1-76]. In this study by Zhao et al., (2015) [76] a putative *WOX2* homolog was isolated and characterized from *Aegilops tauschii*, the donor of D genome of *Triticum aestivum* [76]. The sequence consisted of 2045 bp, and contained an open reading frame (ORF), encoded 322 amino acids [76]. The predicted protein sequence contained a highly conserved homeodomain and the *WUS*-box domain, which is present in some members of the *WOX* protein family [76]. The full-length ORF was subcloned into prokaryotic expression vector pET-30a, and an approximately 34-kDa protein was expressed in *Escherichia coli BL21* (DE3) cells with IPTG induction [1-50]. The molecular mass of the expressed protein was identical to that predicted by the cDNA sequence [76]. Phylogenetic analysis suggested that, *Ae. tauschii**WOX2* is closely related to the rice and maize orthologs [76]. Quantitative PCR analysis showed that *WOX2* from *Ae. tauschii* was primarily expressed in the seeds; transcription increased during seed development and declined after the embryos matured, suggesting that *WOX2* is associated with embryo development in *Ae. tauschii*[76].

Somatic embryogenesis is one of the important pathways for forest tree propagation and genetic improvement [268]. ***WOX8*** is essential for maintenance cell proliferation during both embryonic and post-embryonic development [268]. In this study, *JmWOX8* gene was cloned, containing a 906-bp CDS sequence encoding 301 amino acids [271]. Subcellular localization confirmed its nuclear localization. The amino acid sequence of *JmWOX8* contains a conserved homeobox domain belonging to the Homeodomain super family, and shares > 70% sequence similarity with *WOX8* homologs from other species [271]. The qRT-PCR results revealed *JmWOX8* expression in both embryogenic callus (EC) and non-embryogenic callus (NEC) followed a unimodal pattern, peaking on the 21st day and then dropping sharply by the 28th day[271]. These results suggest that *JmWOX8* plays an important role in the early stage of somatic embryogenesis of *J. mandshurica* [271]. This research work by Li et al., (2025) [271] provides a foundation for elucidating the molecular mechanisms of somatic embryogenesis of *J. mandshurica*[271].

3. Plant Somatic Embryogenesis and Organogenesis

Somatic embryogenesis (SE) is a critical process in plant tissue culture, enabling the regeneration of entire plants from somatic cells rather than through traditional sexual reproduction pathways [80-179, 180-227, 244-246]. SE has significant applications in agriculture, particularly for the propagation of genetically identical plants at a large scale, which is especially valuable for crops that are difficult to reproduce through conventional methods, such as soybeans and bamboo [80-179-227, 244]. This process involves inducing somatic cells to become totipotent, allowing them to differentiate into any cell type and ultimately form a complete plant [80-179-227, 244]. The success of SE is influenced by several factors, including the plant's genotype, the type of explant used, and the composition of the growth medium [80-179-227, 244]. Plant growth regulators, particularly auxins such as 2,4-Dichlorophenoxyacetic acid (2,4-D), play a pivotal role in promoting callus formation and embryo initiation during SE [80-179-227, 244-246]. Additionally, transcription factors like WUSCHEL-RELATED HOMEOBOX (*WOX2*) are crucial for maintaining cellular totipotency and regulating the developmental pathways of somatic embryo [1-79].

Malabadi and coworkers induced and established somatic embryogenesis and plant regeneration in many commercially important plants in India such as grape [80], sugarcane [81], *Catharanthus roseus* [82], *Vigna aconitifolia* [83, 84], *Clitoria ternatea* [85, 87], papaya [88], and mango [89]. In addition to this, Malabadi and coworkers also established an in vitro micropropagation and in vitro seed germination methods for many commercially important orchids in India [135-145, 172] such as *Pholidota pallida* [136], *Xenikophyton smeeanum* [137, 138], *Liparis elliptica* [139], *Aerides maculosum*[140], *Eria dalzelli* [141], *Cymbidium bicolor* [142], *Dendrobium nobile*[143], *Vanda parviflora* [144], *Vanda coerulea*[145], and *Cymbidium elegans* [172]. In addition to this, for an example, Malabadi et al., (2012) [267] also established a successful *in vitro* cloning and plant regeneration of sugar maple (*Acer saccharum*) (Figure-1) at the Department of Agriculture, University of Guelph, Ontario, Canada (Unpublished work) [267].

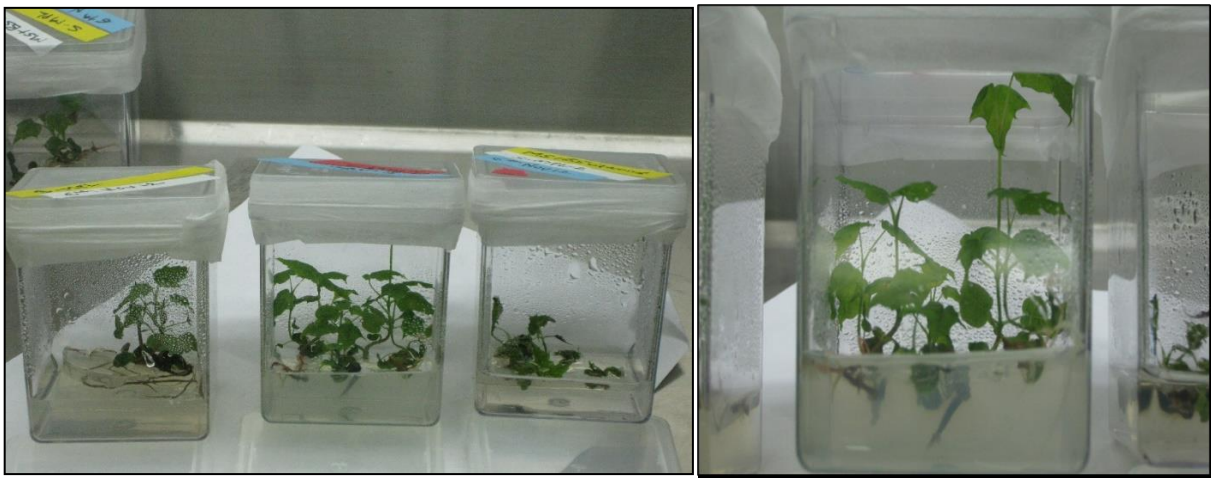

Figure 1 *In vitro* cloning and plant regeneration of Sugar maple (*Acer saccharum*) [Reference, 267]

In case of organogenesis, the explants of selected plant produce plants via callus formation [80-179, 269, 270]. Organogenesis allows for the effective regeneration of new plants from callus [80-179]. Organogenesis is the production of plant organs from a specific tissue in order to develop complete plants [80-179, 269, 270]. It is characterized by being polar, which means clonal propagation of high-value forest trees, medicinal plants, endangered /threatened plant species through somatic embryogenesis has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the uniformity and quality of the nursery stock [80-179, 244-246, 269].

Plant tissue culture is an *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled defined nutritional medium conditions often to produce the clones of plants [80- 227, 244-246]. Plant tissue culture can be used for a wide range of purposes with various applications in research and industry [80- 227, 244-246]. The resulting clones are true to type of selected genotypes and used for the large-scale plant multiplication [80-179-227, 244]. The principle of totipotency, the ability of a single plant cell to regenerate into a whole plant, is the foundation of plant tissue culture [80- 227, 244]. Key to this process is the regulation of growth hormones, particularly auxins, which promote root formation, and cytokinins, which encourage shoot development [80-179-227, 244]. The balance between these hormones determines the regenerative pathway, making them essential for tissue culture success [[80- 227, 244]. Tissue culture technique depends mainly on the concept of totipotentiality of plant cells, which refers to the ability of single cell to express the full genome by cell division [80- 227, 244-246].

The shoot apical meristems are organized pools of undifferentiated or embryonic cells (stem cells) maintained by a dynamic balance between cell division and differentiation [126]. On the basis of study reported by Malabari et al., (2012) [126] it is found that actively dividing and totipotent cells (stem cells) are positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division under *in vitro* conditions leading to a continuous flow of progeny cells (Figure-2, 3, 4) [[126]. These progeny cells (stem cells) under stress conditions (cold/heat) undergo differentiation due to signal activation in cambial region and leading to the embryogenic pathway in conifers (Figure-2, 3, 4) [126]. On the other hand, the rest of the layers (epidermis, cortex region and central pith or medulla) of the transverse thin section of shoot apical meristems of mature trees have induced non-embryogenic tissue under in vitro conditions in conifers [126].

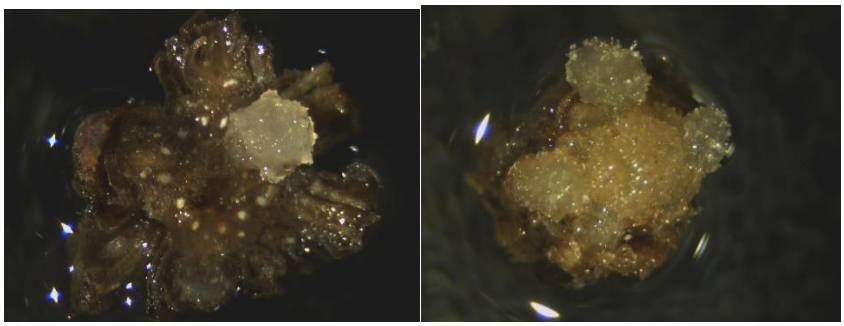

Figure 2 Initiation of embryogenic tissue growth from the cambial region of thin shoot apical dome of mature *Pinus patula*

Cloning of selected superior mature tree is recognized as a powerful tool in forest tree improvement [80- 227]. Somatic cells of many plant species can be cultured and induced to form embryos that are able to develop into mature plants, and termed as somatic embryogenesis [80- 227]. During this somatic-to embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression system [80- 227]. These procedures were accompanied by increased expression of diverse stress related genes; evoking the hypothesis that somatic embryogenesis is an adaptation process of in vitro cultured plant cells [80- 227].

This will also resulted in the micropropagation of a particular tree line under study, and the somatic seedlings could be used for commercial forestry since they have defined genetic characters of superior genotypes [80- 227, 244].

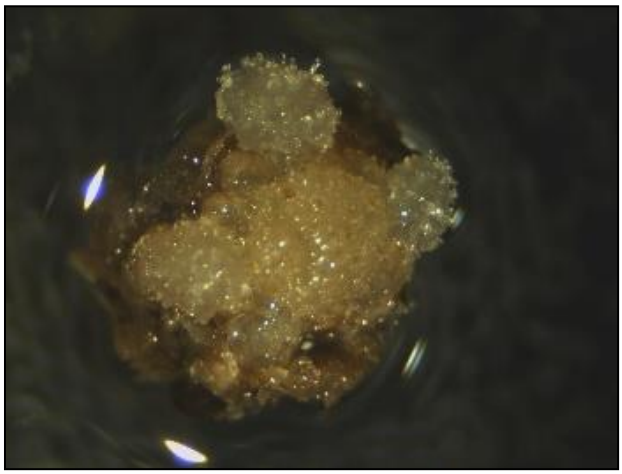

Figure 3 Initiation of embryogenic tissue growth from the cambial region of thin shoot apical dome of mature *Pinus roxburghii*

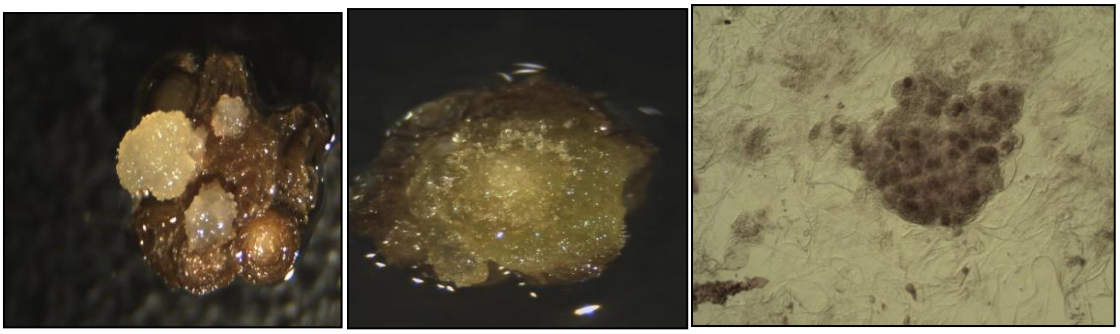

Figure 4 Initiation of embryogenic tissue growth from the cambial region of thin shoot apical dome of mature *Pinus patula, Pinus roxburghii,* and showing early sign of embryonal head formation

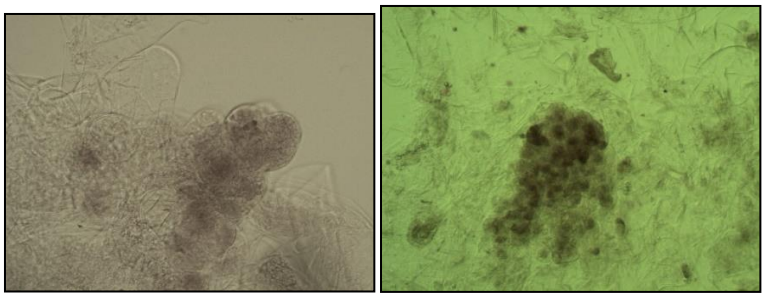

Figure 5 Initiation of cell division showing the early sign of embryonal head formaion in *Pinus roxburghii* and *Pinus patula*

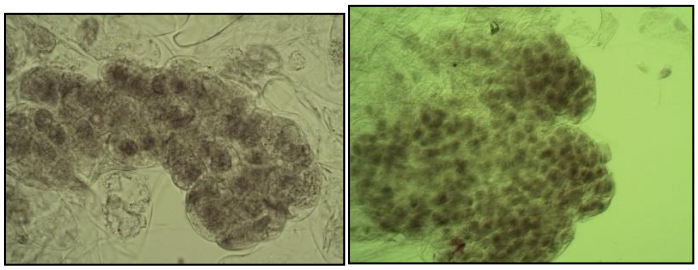

Figure 6 Initiation of cell division showing the embryonal head formaion in *Pinus roxburghii* and *Pinus patula*

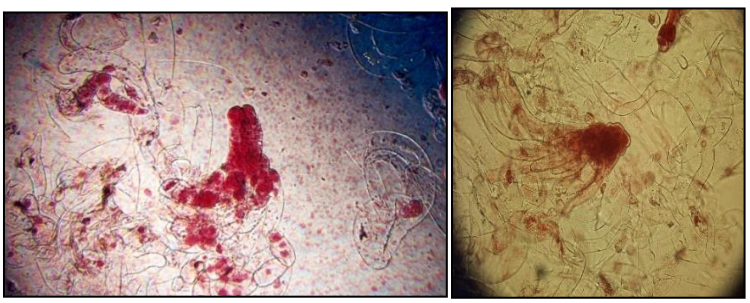

Figure 7 Embryonal head formation in adult tissue of Maritime pine and Scots pine (*Pinus sylvestris*)

Plants continuously maintain pools of totipotent cells in their apical meristems from which root and shoot systems are produced. Most plant organs are formed during the postembryonic stages from the meristems [80- 227]. The shoot meristem is the source of all above ground post-embryonic organs in higher plants [126]. It carries out organ formation by balancing the maintenance and proliferation of undifferentiated totipotent cells (stem cells), and the direction of these cells towards differentiation. In case of pines, the transverse thin layer showed outermost epidermis layer, then internal layer of cortex region, followed by thin cambial region and central pith or medullar region respectively [80-227]. Actively dividing and totipotent cells (stem cells) are positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division lead to a continuous flow of progeny stem cells [80-227]. The activation of the cambial layer cells (stem cells) is one of the important phenomenons for the successful induction of somatic embryogenesis in conifers. This has been achieved in many recalcitrant conifers [126].

Figure 8 Embryonal head formation and a mixture of normal and abnormal somatic embryos derived from the *in vitro* culture of shoot apical domes of *Pinus roxburghii*

The embryogenic cells (stem cells) are very small in size, richly cytoplasmic and actively dividing with a prominent nucleus, rich in starch grains, and very slow in growth under in vitro conditions at the initial stages of the development (Figure-3, 4, 5, 6) [[126]. The slow growth of these cells might be due to reprogramming of the stem cells towards embryogenic pathway. During this phase, cells might be readjusting their metabolic processes due to the stress conditions. After programming of the stem cells towards embryogenic pathway, the growth of cells is regaining to normal [126]. These cells were generally present in small, compact aggregates and showed competence for embryogenesis [126]. These cambial layer cells (stem cells) under stress conditions undergo differentiation and leading to the embryogenic pathway in conifers [126]. On the other hand, the rest of the layers (epidermis, cortex region and central pith or medulla) have induced non-embryogenic tissue in conifers [126]. Microscopic observation of non-embryogenic tissue confirmed loosely arranged, vacuolated and often elongated thin parenchymatous cells with sparse cytoplasm and few starch grains [126]. Such cells did not show morphogenetic competence for somatic embryogenesis in conifers [126]. These cells can survive up to 6 months with multiple subcultures, ultimately leading to death due to the exudation of high phenolic compounds and other factors [126]. The non-embryogenic tissue is often mixed with cambial layer cells, and it is very difficult to separate the embryogenic cells [126].

A simple and inexpensive method to separate embryogenic cells from the mixture of the tissue clumps has been widely adopted in many plant species for the selection of embryogenic cells [126]. In this method, non-embryogenic cells could be largely eliminated by the application of selection procedure and culture of embryogenic cells [126]. Vigorous shaking of the suspension leads to the sinking of larger and denser clumps of embryogenic cells to the bottom of the flask within a few seconds [126]. On the other hand, vacuolated non-embryogenic cells remained in the upper portion [126]. The middle part of the suspension consisting of small clumps of embryogenic cells was used as the inoculums for subculture [126]. Very recently a convenient method has been developed for the selection of embryogenic cells in our lab, and this technique particularly holds good for conifer tissue culture [126]. During this method, a mixture of non-embryogenic tissue and unidentified embryogenic cells should be made into a suspension often mixed with 70% alcohol and sterile antioxidant solution (2ml) [126]. The suspension is centrifuged at the lower speed for about 10-15min resulting in the clear separation of two layers [126]. The bottom layer is composed of minute and small rich embryogenic cells, whereas the upper layer separated as the empty elongated parachymatous non-embryogenic cells [126]. This method is used in our laboratory for the selection of embryogenic cells particularly for the conifer tissue culture experiments [80- 227]. These cells were plated on media under *in vitro* conditions for the induction of somatic embryogenesis in mature conifers [126]. The resulting cultures were composed predominantly of embryogenic cells originated from cambial layer only [126]. If the embryogenic cells were not separated within the stipulated time of the growth, overgrowth of

non-embryogenic tissue could lead to the death of the cells ultimately ends up with the failure of the entire somatic embryogenesis process [126]. At this stage one cannot proceed further and all the cultures will be turning brown in spite of successful initiation of somatic embryogenesis [126]. Identification and separation of embryogenic cells is one of the tedious processes during mature tree cloning of conifers [126]. Therefore, activation of cambial layer is very important in the initiation of embryogenic cultures during the cloning of mature conifers [126]. On the other hand, the thin layer of shoot tip explants cultured under in vitro conditions without activation of the cambial layer also produces a mixture of the callus tissue [126]. This tissue cannot be used for the induction of somatic embryogenesis in conifers since cambial layer cells are not programmed towards embryogenesis [126]. This is very much evidenced by the microscopic observation of the tissue. For the successful programming of the cambial layer cells, activation is very much needed [126]. This activation again depends upon many factors such as type of buds collected, timing of bud's collection, stress conditions, and also signaling molecules [126]. These factors should be optimized before starting cloning experiments of mature trees of a particular pine species under study [126]. During this activation step, formulated media composition only activate the cambial layer cells and inactivate the rest of the layer cells. Therefore, cambial layer cells only produce a callus and rest of the layers failed to produce callus tissue and callus tissue growth is inhibited (Figure-2, 3, 4). This is just like a **magic show** where only **cambial layer** is activated to produce a callus and rest of the layers are inhibited to produce callus tissue (Figure-2, 3, 4).

Another important factor is growth cycle of the particular pine species should be studied before starting mature tree cloning experiments [126]. Furthermore, all the cambial layer cells are not programmed towards embryogenesis under the given in vitro conditions; only a few are programmed and proceed further for the successful embryogenesis [126]. For example, only small no of cells is reprogrammed towards somatic embryogenesis [126]. The further growth of the cells is bloked or arrested due to unknown factors [126]. Under this condition, cells might develop head and suspensor but failed to produce embryos [126]. In another situation, cells are embryogenic but resulted in abnormal embryo formation, embryo-like structures, fake embryos which failed to germinate is very common in conifer somatic embryogenes using buds from mature trees [126]. This also largely depends upon the pine species, and type of the stress conditions used, and signal molecules [126]. Hence activation of the cambial layer is species-specific [126]. In our study the tissue produced from activated cambial layer produced embryogenic cells [126]. Therefore, tissue produced from cambial layer without activation under stress conditions failed to produce embryogenic cells due to the failure of programming of cells towards embryogenesis [126]. This is one of the important steps when we work on cloning of mature conifers [126]. The best way to avoid this problem is to activate the cambial layer of cells and inactivation of rest of the layers (epidermis, cortex, and central medulla or pith) under in vitro conditions for the successful initiation of embryogenic cultures [126]. During activation, only cambial layer of cells produces callus and inhibits callus formation from the rest of the layers (epidermis, cortex region and central pith or medulla) under *in vitro* conditions [126]. The activation of cambial region is a very interesting phenomenon during cloning of mature conifers [126]. This again depends upon the transmission of the signal to the cambial region [126]. As per our previous discussion in one of the review papers, there are many signaling molecules and stress factor, which directly trigger the activation of the cambial layer (stem cells) leading to the initiation of embryogenic tissue [126]. The callus is embryogenic and resulted in the successful induction of the somatic embryogenesis in mature conifers. But till today, how this signal triggers the activation of cambial layer (stem cells) in conifers is largely unknown [126]. Another important question is whether this signal is transmitted from epidermis region to cambial region or central pith or medulla region to cambium region and the entire phenomenon is still unknown in conifers [126].

4. Totipotency of Somatic Plant Cells

The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants [80- 227, 244-246]. It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis [80- 227, 243, 244-246]. This triggers the reprogramming of plant cells into the pathway of embryogenic development (commitment) leading to somatic embryo formation (Figure-5, 6, 7, 8) [80- 227]. These conditions include proper supply of nutrients, source of carbohydrate, pH of the medium, adequate temperature and proper gaseous and liquid environment [80- 227, 244-246]. The controlled conditions provide the culture of explants on a defined nutrient medium with the source of carbohydrate in an environment conducive for their growth and multiplication [80- 227, 243, 244-246]. The disadvantages associated with zygotic explants may be overcome if mass propagation of elite, mature trees can be achieved from vegetative tissue explants, such as secondary needles or apical shoots, because the regenerated plantlets will be uniform and possess elite characteristics from clearly defined parents [80- 227, 243]. The successful cloning of mature trees of conifers was also demonstrated by Bonga and coworkers [205 2219], DR Smith in 1997 [212], Westcot and co-workers [214] and Litz et al., 1995 [194].

Somatic or body cells have the capability to reinitiate an entire ontogenic program, termed somatic embryogenesis [80-227, 232-246]. The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants [80- 227, 232-246]. Therefore, the differentiation of somatic cells is reversible [80- 227, 232-240]. It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis [80-227, 232-246]. This is due to the presence of specific undifferentiated organ cells, the meristems. The activity of meristematic cells is maintained, initiated or stopped by endogenous as well as environmental signals [80- 227, 232-240]. Environmental and endogenous factors together determine the developmental fate of plants through the activation or inactivation of meristems [80- 227, 232-240]. Plant cells attempt to establish a new programme through changes in pH gradients of all cell compartments, arresting differentiated functions, reactivating the compartments, arresting differentiated functions, reactivating the cell cycle and re-organizing gene expression as well as metabolism [80- 227, 232-246].

Under *in vitro* conditions, one or a few somatic cells of the plant or explant have to be competent to receive a signal (endogenous or exogenous). This triggers the pathway of embryogenic development (commitment) leading to somatic embryo formation [80- 227, 232-246]. For a particular genotype or plant, the *in vitro* forms of somatic embryogenesis, the optimum conditions (potential, competence, induction, and commitment) have to be experimentally optimized [80-227, 232-246]. Although *in vitro* somatic embryogenesis is practiced in many tissue culture laboratories throughout the world using many conifer species, genotypes and explants, the biological background of the process is still largely unknown and not well studied [80- 227, 232-246]. Therefore, we still do not know how and why competence or commitment is achieved by a somatic cell or what is the real trigger (signal) initiating embryo development [80- 227, 232-240]. It was presumed earlier that the potential of somatic embryogenesis is determined at the level of the genotype, which is clearly proved by the successful transfer of the embryogenic capacity between embryogenic and recalci- trant genotypes via sexual crossing [80- 227, 232-240].

Recalcitrance could be resolved by optimizing *in vitro* growth conditions of plants or by proper explant selection. Genetic determinants therefore, may only serve to define the conditions when and where embryogenic competence can be expressed [80- 227, 232-240]. Thus, embryogenic potential is largely defined by the developmental programme of the plant as well as by environmental cues or stimuli [80- 227, 232-246]. Unlike carrot or alfalfa, where somatic embryogenesis can develop on all organs of seedlings, in conifers, embryogenic competence is restricted to certain tissues of a given genotype. Tissue culture studies supported the view that there exists a kind of gradient in the embryogenic response among various plant organs [80- 227, 232-240-244-246]. The embryogenic potential is highest in tissues with embryonic origin and decreases in other explants of the same plant species. Therefore, immature zygotic embryos are genetically programmed to produced embryos. On the other hand, somatic cells or body cells are not genetically programmed towards embryo formation. But somatic cells can be reprogrammed towards somatic embryogenesis under stress conditions, influence of media and growth regulators, time of the bud collections, and many other factors. However, even if embryogenic competence seems to be lost in somatic plant cells, it can potentially be regained [80- 227, 232-240, 244-246]. In this indirect somatic embryogenesis pathway, an intermediate callus formation phase is required in order to express embryogenic potential [80- 227, 232-240]. Therefore, differentiated plant cells do not lose their developmental potential during normal development but retain plasticity, that is, they are capable of dedifferentiating and acquiring new developmental fates (the so-called universal totipotency of plant cells [80- 227, 232-240-246].

5. Story of Pine Tissue Culture in India (1960-2000)

In India, pine tissue culture research has started in 1960-1970 under the guidance of Professor RN Konar funded by the Government of India project at the University of Delhi, New Delhi, India and successfully initiated the conifer tissue culture research and published in *Physiologia Plantarum* in 1974 [199, 200, 232-237-262]. Maheshwari P, Nagamani R and Konar RN have also published the results of the pine tissue culture in Botanical Monograph. CSIR Publications, New Delhi, India in 1971 [199, 200, 232-234-262]. But during initial years, there are many failures in establishing the pine tissue culture work in India.

Professor K. Nataraja was also worked (1960-1965) under the guidance of Professor RN Konar interested in tissue culture studies particularly induction of somatic embryogenesis in plants at the University of Delhi, New Delhi, India [199, 232-234-262]. After that, Professor K. Nataraja has also started working on induction of somatic embryogenesis in plants and other tissue culture studies. Professor K. Nataraja has guided more than 28 Ph. D students including Dr. Ravindra B. Malabadi at the Department of Botany, Karnatak University, Dharwad, Karnataka State, India. In addition to this, Professor K. Nataraja also guided M. Phil students in the plant tissue culture studies. Nataraja K and RN. Konar at the Department of Botany, University of Delhi, New Delhi, India were Indian botanists who published influential

research in the 1960s and 1980s on experimental embryogenesis in the plant *Ranunculus sceleratus* L., demonstrating how plant tissues could be used to form plantlets and contribute to advances in plant tissue culture [198, 250-252-256]. Double haploid technology (DH) is an essential tool in plant breeding, enabling the rapid production of homozygous lines. However, doubled haploids (DH) were not highly relevant in plant breeding until researchers at the Department of Botany in the University of Delhi, India, reported a major breakthrough in the production of haploids from another culture in *Datura innoxia* (Guha and Maheshwari, 1964, 1966) [269, 270]. Their research revolutionized the use of doubled haploid (DH) technology in plant breeding worldwide [269, 270].

This long legacy (1960-2000) of the research work of Professor RN Konar and Professor Pramod Tandon, NEHU, Shillong, Meghalaya, India (1996-2000) has led to the successful initiation of embrygenic culture from the shoot apical meristem culture of mature trees of *Pinus kesiya* by Malabadi and other co-workers [98-102]. Later, Professor Pramod Tandon and co- workers in India successfully initiated, established and plantlet regeneration from different explants (shoot apical meristem, secondary needles, mature zygotic embryo and immature zygotic embryos) of mature trees of *Pinus kesiya* [180-184, 172, 186, 188, 189-192, 193, 185]. This project during 1996-2000 was funded by the Department of Biotechnolgoy (DBT), Government of India, New Delhi, India under the guidance of Professor Pramod Tandon, Plant Biotechnology Laboratory, North Eastern Hill University (NEHU), Shillong, Meghalaya State, India [180-184, 172, 186, 188, 189-192, 193, 185]. Dr. Sartia Arya and co-workers, Scientist at Arid Forest Research Institute (AFRI), New Pali Road, Jodhpur- 342005, Rajasthan State, India and Tissue Culture Laboratory, Division of Genetics and Tree Propagation, Forest Research Institute (FRI), Dehradun-248006, Uttaranchal state, India has also initiated and established embryogenic cultures and plantlet regeneration of *Pinus roxburghii* in India [219-225]. Furthermore, more systematic, and refined study has been done successfully by Dr. Ravindra B. Malabadi and co-workers established the pine tissue culture studies in *Pinus kesiya*, *Pinus roxburghii* and *Pinus wallichiana*, *Pinus caribaea* and *Pinus gerardiana* in India [80-179]. Therefore, knowledge and research work of Professor RN Konar, Professor P. Maheshwari, and Professor K. Nataraja and Professor Pramod Tandon, (1960-2000) has helped for the establishment of pine tissue culture studies in India [80- 227]. In addition to this Government of India has recognized this outstanding contribution of Professor Pramod Tandon for the initiation, establishment and successful regeneration of pine tissue culture in India [180-184, 172, 186, 188, 189-192, 193, 185]. Professor Pramod Tandon is an Indian Plant Biotechnologist and academic. He is a former Professor of Botany and Vice-Chancellor of North-Eastern Hill University (NEHU), Shillong, Meghalaya, India and Chief Executive Officer of Biotech Park, Lucknow. UP, India. The Government of India has awarded Professor Pramod Tandon the fourth highest civilian honour Padma Sri in 2009, for his outstanding contribution to plant science, Professor Pramod Tandon is a Fellow of the National Academy of Sciences, India, Indian Botanical Society, Indian Botanical Society, Linnean Society of London, and International Society of Environmental Botanists and served as a member of many Academic bodies and National Task Forces including Scientific Advisory Committee to the Cabinet, GOI. The other pine tissue culture work in India was also done by Dr. Sarita Arya, Dr. ID Arya, Dr. RK Kalia, and Dr. Rajani S. Nadagouda [220-230]. Further the research work in pine tissue culture work done by Dr. Pramod k. Gupta (USA) and shri Mohan Jain (Finland) is also acknowledged [220-230].

In addition to this, Dr. Chittaranjan R. Deb and Professor Pramod Tandon in India have also initiated, established embryogenic cultures and successful regeneration of plants from shoot apical meristem cultures of mature trees of *Pinus kesiya* [180-185, 188]. Chittaranjan R. Deb and Tandon (2002) reported successful initiation, establishment, and plant regeneration from secondary needles of mature trees of *Pinus kesiya* [180-185, 188]. Deb and Tandon (2004) also reported successful regeneration of plantlets from different explants (shoot apical meristem, secondary needles, immature zygotic embryo, zygotic embryo) of mature trees of *Pinus kesiya* [180-183-184, 188]. In addition to this, Dr. Hiranjit Choudhury and co-workers in India also reported successful initiation, establishment ad plantlet regeneration from different explants (shoot apical meristem, secondary needles, immature zygotic embryo, zygotic embryo) of mature trees of *Pinus kesiya* [187, 188, 189-192, 193, 194]. Dr. Sanghamitra Purkayastha [186] also reported the developmental physiology and biochemistry of somatic embryogenesis from different explants in *Pinus Kesiya* Royle Ex. Gord [186].

Indian plant biotechnologists established a legacy, history and championship in pine plant tissue research work which is evidenced, demonstrated and acknowledged by published research work in national and international journals since 1960 to 2010 [180-184, 172, 186, 188, 189-192, 193, 185, 219-228]. However, in spite of a legacy, history and championship, commercialization of pine tissue culture work in India is still facing problems and major bottleneck due to the many reasons, lack of financial funding for this work, lack of laboratory resources, no job market for pine tissue culture work, shift in the research area, no one is interested in pine tissue culture due to low commercial value, low yield of plantlets, high risk of failure, waste of time in achieving project goals, end result is not worth, shifting cultivation for more exotic and commercially important tree species like Sandal wood (*Santalum album*), Teak (*Tectona grandis*), red cedar, rosewood (*Dalbergia latifolia*), sal (*Shorea robusta*), banyan tree, oak, birch, aspen, elm, maple, bamboo, and palm, and already Government of India has supported and funded the pine tissue culture work for long period of time

(1965-2000). Another major problem in India is that most of the senior Scientists those who have wet laboratory experience in pine tissue culture work were retired and this rich hidden knowledge of pine plant tissue culture has not been transferred to future younger generation. Therefore, there is a major gap of almost 25 years in pine plant tissue culture studies in India. At this point of time, pine tissue culture work in India is dead and remained as the academic research of the experimental model and failed commercialization. Furthermore, the protocol also suffered due to low plantlet regeneration, pine tissue culture is time consuming, and very slow. There is a major shift in research funding in India for more current issues like accumulation of microplastic pollution, hydroponics, application of 3D printing technology, plant based wound healing, plant-based leather production, nanotechnology, bioinformatics, cancer biology, genome editing *CRISPR/Cas9*, vaccine research, biodiesel and Cannabis research. Furthermore, plant tissue culture is also used for the preservation and conservation of Indian endangered plant species. More research work is funded in the micro propagation of commercially important orchids, medicinal plant and molecular docking studies. Recently India has legalized Industrial *Cannabis sativa* (hemp), and more funding is available from Government of India and private companies [175-177]. Therefore, Cannabis sativa research is of prime importance in India and funding is available [175-177]. This new ray of hope opened door for many Indian biotech companies for the commercialization of hemp products in the Indian market. This also creates more opportunities and job market in India. One of the success story in **2025** is that India has released biodegradable plastic by corn starch in market and more demand has been observed in Indian market [229-231]. In 2025, Nandini milk company in Bengalore, Karnataka State, India is the first in India using corn based biodegradable plastic for packaging and supplying milk [229-231].

6. The concept of Reprogramming Somatic cells

The somatic cells of the plants cultured under *in vitro* conditions are reprogrammed towards somatic embryogenesis [80-119]. The concept of reprogramming somatic cells is only possible due to some of the stress related factors, then interaction of growth hormones, selection of proper explants, the particular stage growth explants. and role of signalling molecules [80-119].

According to the study conducted by Malabadi et al., (2012) [124] pine bud break (burst) timing is very important factor, which influences the in vitro cloning mature trees of conifers [124]. The timing of bud break of pines is also varied from one location to another location area within the country or from one country to another country within the same continent or different geographical location throughout the world. Survival of pine species depends on synchronization of their annual growthdormancy cycle, bud break or burst with local climate [124]. Bud burst depends on dormancy release by chilling or heats due to increase in the day temperature, and accumulation of thermal time above a species-specific threshold [124]. The buds collected immediately after the bud burst were found very responsive for the in vitro cloning of *P. kesiya, P. roxburghii and P. wallichiana, P. patula,* and *P. sylvestris* [124]. This might be due the activation of the apical meristematic cells showing active growth of shoots in most of the conifers. The active dividing and totipotent cells might be positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division lead to a continuous flow of progeny cells [124]. According to the study conducted by Malabadi RB et al., (2012) [124] these cambial layer cells under stress conditions undergo differentiation and leading to the embryogenic pathway in conifers [124].

In vitro tissue culture conditions expose the explants to significant stresses, as they are removed from their original tissue environment and placed on synthetic media containing non-physiological concentrations of growth regulators, salts and organic components. Wounding itself is a significant signal in the induction of dedifferentiation [124]. Stresses not only promote dedifferentiation, but also can be used to induce somatic embryo formation [124]. Wounding, high salt concentration, heavy metal ions or osmotic stress positively influenced somatic embryo induction in diverse plant species. These procedures were accompanied by increased expression of diverse stress related genes; evoking the hypothesis that somatic embryogenesis is an adaptation process of in vitro cultured plant cells [124].

Malabadi et al., (2012) [124] reported that plants continuously maintain pools of totipotent cells in their apical meristems from which root and shoot systems are produced [124]. Most plant organs are formed during the postembryonic stages from the meristems [124]. The shoot meristem is the source of all aboveground post-embryonic organs in higher plants. It carries out organ formation by balancing the maintenance and proliferation of undifferentiated totipotent cells, and the direction of these cells towards differentiation. The shoot apical meristems are organized pools of undifferentiated or embryonic cells maintained by a dynamic balance between cell division and differentiation [124]. In case of pines, the transverse thin layer showed outermost epidermis layer, then internal layer of cortex region, followed by thin cambial region and central pith or medullar region respectively [124]. The actively dividing totipotent cells are positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division lead to a continuous flow of progeny cells [124]. These cambial layer cells under stress

conditions undergo differentiation and leading to the embryogenic pathway in conifers. On the other hand, the rest of the layers (epidermis, cortex region and central pith or medulla) have induced nonembryogenic tissue in conifers [124].

Signal molecules regulating embryo development have been described in angiosperms, but very little is known about somatic rejuvenation in conifers [123]. Recent studies on cloning of mature conifers provide new perspectives on signal molecules on cellular dedifferentiation into the embryogenic pathway. According to the study conducted by Teixeira da Silva JA and Malabadi RB [123] the signal molecules such as butenolide, calcium ions, salicylic acid, antioxidants, amino acids, triacontanol and 24-epibrassinolide all play an important role in the conversion of somatic cells into an embryogenic pathway in many recalcitrant pines [123]. This constitutes a major breakthrough in forest biotechnology with many practical applications in clonal forestry [123].

The induction of somatic embryogenesis using shoot apical thin layers has been successful by Malabadi and co-workers in few conifers such as *Pinus roxburghii* [98-102, 104], *Pinus kesiya* [95], *Pinus wallichina* [97], *Pinus patula* [105-113], *Pinus sylvestrus* (Scots pine) [121, 122], and Maritime pine, *Pinus pinea* [119]. The somatic cells (cells from the bud culture) are not genetically programmed towards somatic embryogenesis [80-119]. Somatic cells are re-programmed towards somatic embryogenesis pathway under stress conditions/growth harmones applications/media formulations applied. The best explanation is that during reprogramming, all the cells are not programmed in pines. However, only few cells are progrmmed and proceed towards altered pathway that is somatic embryogenesis. Sometimes, the growth of these cells is arrested or blocked the pathway. Rest of the cells are not programmed and produce non-embryogenic callus. Only cambial cells containing stem cells are reprogrammed towards embryogenic pathway. Suppose, in an example, small percetage of cells (5-20%) are reprogrammed towards embryogenic pathway, these cells proceed to some extent and will not survive due to high percentage of non-embryogenic tissue growth. On the basis of studies, it is found that epidermis and cortex laters produced non-embryogenic tissue (80%) and this has created a major problem and inhibited the further growth of embryogenic tissue growth by small percentage of reprogrammed cells towards somatic embryogenesis. These reprogrammed cells towards somatic embryogenesis should be identified, separated, and cultured immediately (Figure: 5, 6, 7, 8). Furthermore, this altered pathway might be blocked or arrested or inhibited by several factors. Therefore, these cells will not proceed further. During these altered pathways, cells can produce head, suspensor, and like embryo-like structures, abnormal embryos, fake embryos and failed to germinate (Figure-8). This is a common phenomenon in pines. Sometimes, growth of cells is arrested leading to the failure of embryo formation. In another case, cells are programmed, proceeded to few stages and further growth is arrested/blocked and failed to produce somatic embryos and resulted in embryo-like structures and fake embryos (Figure-7). During altered pathway, cells are forced to form the embryo like structures, fake embryos, and somatic embryos. Therefore, there is a mixture of embryos formed. Only healthy somatic embryos have produced plantlets. Each pine species is genetically different and cells behavior on particular cultured media is different. Therefore, there are many external and internal factors govern the process. Hence, stress conditions applied, time of the bud collections and effect of particular growth hormone, used and media formulations plays an important role in inducing reprogramming cells towards somatic embryogenesis in pines. Each pine species in different geographical region is genetically different. In addition to this, re-formulation of the media, with new precursor moleculs inducing somatic embryogenesis should also be applied and studied. There are two types of precursor molecules, natural precursor and synthetic precursor molecules should also be studied. However, this is a time-consuming studies leading to the long-term research. These treatments might induce 70-80% of cells towards somatic embryogenesis and successful healthy plantlet regeneration. During last 25 years, there are many natural precursor and synthetic precursor molecules have been identified. However, there are no studies reported till today on these molecules triggering reprogramming plant cells towards somatic embryogenic pathway and breaking the recalcitrance.

Recalcitrant is very common in many plant species under *in vitro* conditions. But many recalcitrant plant species have been cloned successfully via organogenesis or somatic embryogenesis. This could be possible only by reprogramming the cell pathway towards somatic embryogenesis. There are many signalling molecules which can re-programme the dipoloid (somatic cell) cell to somatic embryogenesis. The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants. Many of the recent studies showed that signaling molecules such as butenolide, calcium ions, salicylic acid, antioxidants, amino acids, triacontanol, melatonin, and 24-epibrassinolide all play an important role in the conversion of somatic cells into an embryogenic pathway in many recalcitrant pines, and tree species. It can be best demonstrated in an *in vitro* system where somatic plant cells can regain their totipotency and are capable of forming embryos through the developmental pathway of somatic embryogenesis. Another important factor is that one has to develop natural or synthetic precursor molecules which can trigger and reprogramming of the cells towards somatic embryogenesis. These precursor molecules can break the recalcitrant nature of plant cells and resulted in successful organogenesis or somatic embryogenesis. However, the interaction studies of new precursor molecules with plant cells under *in vitro* conditions is a long-term study which needs funding, good laboratories facilities, well trained scientists particularly in the field of somatic

embryogenesis and challenging too. Sometimes, these studies might end up as experimental models and commercialization is still a bottleneck. Therefore, commercialization of plant tissue protocols in many plant species is a major problem and challenging too. Artificial neural networks (ANNs) and machine learning (ML) are widely used in science and technology, and have been successfully applied in cannabis plant tissue cultures [271]

Another best way is to culture only central cambial layer cells and remove the outer epidermis and cortex layers. In another development, one should also apply modern cell sorting techniques to separate the embryogenic cells. Identification and seperation of embryogenic cells might help to solve problems. **Artificial Intelligence** (AI) and machine learning should also be applied in order to identify these cells, separated and cultured [271].

7. *Pinus Roxburghii*: Induction of SE from shoot apical domes of Mature trees

7.1. *Pinus Roxburghii*: Initiation of embryogenic tissue

According to method described by Malabadi et al., (2012) [3], shoot apical domes from mature trees (14- years old) of *Pinus roxburghii or Chir pine* of 3 genotypes (PR11, PR105, and PR521) were collected from the Western Ghat Forests, India (140 5' to 15 0 25' N latitude and 74 0 45' to 76 0 E longitude with an average rainfall of 80 cm.). They were cleansed with 1% Citramide (Sodium hypochlorite 3.5%) for 5 min and rinsed thoroughly with sterilized distilled water. These were surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4-times with sterile double distilled water. Transverse-thin sections of approximately 0.5-1.0 mm thick were cut using sharp sterilized blade or scalpel from shoot apical domes (upper part with 2 to 3 sections only) for the initiation of embryogenic tissue. These shoot apical dome sections were cultured individually on full strength (Inorganic salts) DCR (Gupta and Durzan, 1985) medium containing 0.2 g l-1 polyvinyl pyrollidine (PVP), 2.0 g l-1 Gellan gum (Sigma), 30 g l-1 maltose (Analar, Sigma) and 0.3 % activated charcoal (Sigma without growth regulators. The cultures were raised in 25 mm X 145 mm glass culture tubes (Borosil) containing 15 ml of medium. These cultures were incubated in dark at 40 C for 3 days. Thin apical dome sections after incubation in dark at 40o C for 3 days were subcultured on full strength DCR medium supplemented with 22.62 µM 2, 4-D, 26.85 µM NAA, and 8.87 µM BA (initiation medium) (Malabadi and Nataraja, 2006ab). The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The media were then sterilized by autoclaving at 121oC and 1.05 kg/cm2 for 15 min. L- glutamine and casein hydrosylate were filter sterilized and added to the media after it had cooled to below 50° C. All the cultures were maintained in the dark at 25 ± 2 o C with 55- 60% relative humidity. All the cultures were maintained in the dark at 25 ± 2° C for four weeks. Embryogenic tissue was initiated according to our previous existing protocols (Malabadi et al., 2004; Malabadi and Nataraja, 2006ab; Malabadi and van Staden, 2006). For the initiation of non-embryogenic tissue, thin sections of shoot apical dome without cold- pretreatment subcultured on the initiation medium (Malabadi and Nataraja, 2006ab) were served as the control. They were also maintained in dark for four weeks.

7.2. *Pinus roxburghii*: Maintenance of embryogenic tissue

The embryogenic tissue showing proembryonal masses was again subcultured onto maintenance medium. The full-strength DCR (Gupta and Durzan, 1985) basal medium containing 60 g l^{-1} maltose, 2 g l^{-1} Gellan gum supplemented with 2.26 µM 2,4- D, 2.68 µM NAA and 0.88 µM BA (maintenance medium) was used for this purpose in accordance with our existing previous protocols (Malabadi and Nataraja, 2006ab). On the maintenance medium, embryonal suspensor masses were cultured for 30 days with two subcultures. The presence of embryonal masses was determined by morphological and microscopic observations. The non- embryogenic cultures (control) were also subcultured on the maintenance medium and maintained in dark condition.

For the following experiments of WUSCHEL (*WOX2*) (Palovaara and Hakman, 2008) gene expression studies, two types of plant material, 1) embryogenic tissue initiated by cold-pretreatment, and 2) control (non- embryogenic tissue) induced without cold pre-treatment have been used for the isolation of total RNA. For total RNA isolation, one-gram fresh wt of embryogenic tissue and control (non-embryogenic tissue) were then placed in a cryostorage vial partially immersed in liquid nitrogen. Ten vials of plant tissues (embryogenic tissue and non-embryogenic tissue-control) were collected. Frozen tissues were stored at -70°C until further analyses were performed.

7.3. *Pinus roxburghii*: RNA preparation and cDNA synthesis

Mature bud-derived cell lines and all other tissues analyzed were collected in duplicate, and immediately frozen in liquid nitrogen. Frozen samples of embryogenic tissue and control (non-embryogenic tissue) derived from the apical shoot buds of *Pinus roxburghii* of 3 genotypes (PR11, PR105, and PR521) were ground in a mortar and pestle with liquid nitrogen. Approximately 0.5 g of fresh frozen tissue from each sample was ground in a mortar and pestle with liquid nitrogen. Total RNA was isolated according to the modified method of Chang et al. (1993). To remove residual genomic

DNA, 25 µg of RNA was treated with TURBO-DNase™ (Ambion, Austin, TX, USA). cDNA was generated from 1 µg of DNase-treated RNA using the Superscript II RT system (Invitrogen, CA, USA) according to the manufacturer's protocol and previously reported protocols (Park et al. 2009 and Malabadi et al., (2012) [3]. Each reaction was run in duplicate, generating two independent cDNA samples for each RNA sample isolated from 3 genotypes (PR11, PR105, and PR521) of *P. roxburghii* as reported by Malabadi et al., (2012) [3].

7.4. Cloning of *P. roxburghii WOX2*

For the isolation of the *WOX2* gene, following gene specific primers were designed based on homologous sequences publically available, including *PaWOX2* (*P. abies WOX2*, Acc. AM286747), *PtWOX2* (*P. taeda WOX2*, Acc. DR693345; Cairney et al. 2006) and *Pc WOX2 Pinus contora WOX2*. GenBank Acc no: HM852976.1; Park et al. 2009[26]). The gene specific primers were custom synthesized and used for the PCR amplification of the *PR WOX2* gene using cDNA as the template. Gene alignment was done using Bio Edit programme. Following PCR amplification from *P. roxburghii* cDNAs, the resulting amplicons were cloned into the TOPO-TA cloning vector (Invitrogen) (Park et al. 2009 [26]; Malabadi et al., (2012) [3]. The gene *WOX2* was confirmed by the DNA sequence analysis. (Forward: 5'-ATG GCC GAG GGT CAA TCC ACC ATG A-3'); (Reverse 3' CTT GCC AGG ATG CTG AGG GAT A-5').

For further characterization of the embryogenic tissue, Malabadi et al., (2012) [3] have developed a potential molecular marker on the basis of the expression of one of the important transcription factors, *WOX2* (WUSCHEL homeobox 2) in the embryogenic tissue of *P. roxburghii*. Transcription factor, *WOX2* (WUSCHEL homeobox 2) was strongly expressed in the embryogenic tissue but not in non-embryogenic tissue (control). In Malabadi et al., (2012) [3] study, embryogenic tissue was developed under cold stress conditions. On the other hand, non-embryogenic tissue was induced without cold treatment. Expression of *WOX2* (WUSCHEL homeobox 2) in the embryogenic tissue of 3 genotypes (PR11, PR105, and PR521) of *P. roxburghii* clearly confirmed its involvement in the somatic embryogenic pathway and might be directly related to stress conditions. Therefore, stress conditions always induce somatic embryogenesis in many plant species which also directly related to the expression of genes. This is the first evidence of expression of *WOX2* in *P. roxburghii* and could be used as marker for the identification of embryogenic tissue in pines. In gymnosperms, no *WUS* homolog was found (Palovaara and Hakman, 2008) and as such it is believed that the *WOX* and WUS genes have not diverged in some species (Kiselev et al. 2009; Park et al. 2009).

An interrogation of the *WOX* family members in gymnosperms obtained from the public databases identified that PrWOX2 has 80% similarity to *P. abies PaWOX2* and is almost identical to other pine species sequence such as *PsWOX2* of *Pinus sylvestris* (99% homology) *and PtWOX2* of *P. taeda* (100% homology) (Park et al. 2009), and *PrWOX2* of *P. roxburghii* (Malabadi et al., 2012) [3]. These findings are consistent with previous findings (Malik et al. 2007, Palovaara and Hakman, 2008, Palovaara et al. 2010) which suggested that *WOX2* may be a potential marker to predict the embryogenic potential of spruce and *Brassica* cultivars (Park et al. 2009 [26]; Malabadi et al., (2012) [3]. Therefore, on the basis study by Malabadi et al., (2012) [3] further strengthens the concept of cloning of mature trees of pines. *PrWOX2* could be used as a potential genetic marker for the identification of the embryogenic cultures, which is just an added advantage for developing SE protocols for recalcitrant pines (Malabadi et al., (2012) [3].

8. Lodgepole pine (*Pinus contorta*)

8.1. Cloning of *PcWOX2* and *PcHAP3A*

Park et al., (2009) [26, 245] reported SE propagation system for MPB-resistant lodge- pole pine, several families displaying varying levels of resistance were selected for experimentation involving shoot bud and immature seed explants [26]. In bud cultures, eight embryogenic lines were induced from 2 of 15 genotypes following various treatments [26]. Park et al., (2009) [26] also indicated that genotype had an important influence on embryogenic culture initiation, and this effect was consistent over time [26, 245]. These lines were identified by microscopic observation and genetic markers. Despite the abundance of early somatic embryos, the cultures have yet to develop into mature embryos [26, 245]. In contrast, immature zygotic embryos (ZEs) cultured from megagametophytes initiated SE at an early dominance stage via nodule-type callus in 1 of 10 genotypes [26, 245]. As part of the study by Park et al., (2009) [26, 245], putative embryogenesis-specific genes, *WOX2* (WUSCHELL homeobox 2) and *HAP3A,* were analyzed in cultures of both shoot bud explants and ZEs [26]. On the basis of these analyses, Park et al., (2009), postulated that *PcHAP3A* was expressed mainly in callus and may be involved in cell division, whereas *WOX2* was expressed mainly in embryonal mass (EM)-like tissues [26, 245]. The findings from this study, based on molecular assessment, suggest that the cell lines derived from bud cultures were truly EM [26, 245]. Moreover, these experimental observations suggest that *PcWOX2* could be used as an early genetic marker to discriminate embryogenic cultures from callus [26, 245].

In an attempt to isolate the putative *WOX2*-ortholog from *P. contorta* EM culture, Park et al., (2009) [26, 245] designed the primers (forward: 5' -ATGGCCG AGGGTCAATCCACCATGA-3'; reverse: 3'-CTACTT GCCAGGATGCTGAGGGATA-5') based on homologous sequences publically available, including *PaWOX2* (*Picea abies WOX2*, Acc. AM286747) and *PtWOX2* (*Pinus taeda WOX2*, Acc. DR693345; Cairney et al. 2006) [245]. For the *HAP3A* ortholog, primers (forward: 50-TTGTAGGT ATGATGTCCGAAGTTGG-30; reverse: 30-CCATCAGTCT ATTCTCAACAGTTTA-50) were designed from homologous sequence regions of *Picea glauca HAP3A* (Acc. DR548381) and a cDNA expressed sequence tag (EST) of *P. taeda HAP3A* (Acc. DT627043) [26, 245]. Gene alignment was performed with the BioEdit program. Following PCR amplification from *P. contorta* cDNAs, the resulting amplicons were cloned into the TOPO-TA cloning vector (Invitrogen) and sequence was confirmed [26, 245].

8.2. *Pinus contorta*: Absolute real-time qRT–PCR

Park et al., (2009) [26, 245] reported that Critical threshold (ct) values for *PcWOX2* and *PcHAP3A* were quantified in triplicate with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on an Mx3000P Real-Time PCR System (Stratagene, CA, USA) [26]. The forward and reverse primers for RT–PCR analysis were dtWX2-F (50-CCACAGCAGCGATCCACAACGACCC-30) and dtWX2-R (30-AGCGATGCCGGACGGATGCAATG GG-50) for PcWOX2, and dtHAP3-F (50-GCTGTGAGAGA GCAAGATAGGTTCA-30) and dtHAP3-R (30-CACTGGTG ATGAAGCTTATGTACTC-50) for PcHAP3A [26, 242]. Negative (distilled water) and no-template (total RNA) controls were included in each run [26, 245]. Thermocycler conditions for all PCR reactions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s [26, 245]. Absolute quantification of *PcWOX2* and *PcHAP3A* copy number in each cDNA sample was determined using a standard curve and normalized per microgram of total RNA [26, 245]. The standard curve was generated with purified PCR product-obtained gene-specific primers for *PcWOX2* and *PcHAP3A* that were serially diluted from 101 to 106 copies [26, 245]. The corresponding copy number was calculated as previously described by Whelan et al. (2003) [241].

According to the observations, Park et al., (2009) [26, 245] postulated that *PcHAP3A* is expressed mainly in the callus and may be involved in cell division [26]. Therefore, *PcHAP3A* is unable to differentiate between embryogenesis and NE tissue (callus), as both are actively dividing tissues [26, 245]. The findings from this study by Park et al., (2009) [26, 245] based on the molecular assessment, suggested that the cell lines derived from bud cultures were truly embryogenic, not just cells that imitate embryogenic cultures (EC) in morphology [26]. Moreover, these experimental observations also suggest that *PcWOX2* could be used as an early genetic marker to discriminate EC from callus. In summary, Park et al., (2009) [26, 242] suggested that the use of cell stress, in combination with other culture conditions, has the potential to induce SE in culture of mature gymnosperms, which are notoriously recalcitrant [26]. However, a mechanism to further the development to a mature stage is also warranted [26, 245]. Understanding the underlying molecular and biochemical mechanisms that underpin these processes will aid in our understanding of the reprogramming process(es) that occur during SE [26, 245].

Pinus contorta putative wuschel homeobox protein *WOX2* (wox2) mRNA, com - Nucleotide - NCBI (nih.gov) (Park et al., 2009 [26, 245]. GenBank: HM852976.1 564 bp linear mRNA. Accession: HM852976.1: GI: 312861912 [245].

- 1 atggccgagg gtcaatccac catgagcacc aggtggaatc caacgaaaga acaaatagac
- 61 ttcctggagg ccatgtacag tcaagggatc cgcactccca gtgccgatca aatagaggaa
- 121 atcgccagtc gactgcgaat gtatggaaat attgaaggga agaatgtgtt ttactggttt
- 181 caaaaccata aagctcgcga gaggcagagg cagagacaag agagagtagc gttcgtcaat
- 241 cagtttcatc aaccacctgg cttcgcagaa cttctccctc cacagcagcg atccacaacg
- 301 acccttttcaa aggctggttc ttcaatggca cccagggagg attacaactt ccagcattca
- 361 catgacagtt taaatgaacc tcagacgctg gagctattcc cattgcatcc gtccggcatc
- 421 gctgaataca gatctgaacc agtaggcaca tttggattgc aaggctcgat gaacgagaat
- 481 attgatgaac aaaacgaccc aagatcaggc ggggggcatt ttcatcattt tttccatttt
- 541 atccctcagc atcctggcaa gtag [Reference-**245**].

9. Conclusion

Transcription factors (TFs) are crucial for the transcriptional and post-transcriptional control of genes involved in response to environment stress response. *WOX* gene family was first discovered in *Arabidopsis thaliana* in 1996, with its essential role in shoot and floral development. *WOX* gene family play an important role in the whole plant's growth and development, such as in the stem, embryo, root, flower, and leaf. Many studies using *Arabidopsis* as a model plant have increased our knowledge of the function of embryogenesis-related genes in angiosperm. *WOX2* increases the expression of the auxin transporter *PIN1* gene to regulate stem cell organogenesis in *Arabidopsis*. *PRWOX2* has been

expressed in embryogenic cultures initiated from apical buds of mature trees of *Pinus roxburghi*. *PpWOX2* (*Pinus pinaster*) overexpression enhanced somatic embryogenesis and plant organ formation in *Arabidopsis* transgenic seedlings. Somatic embryogenesis (SE) is a critical process in plant tissue culture, enabling the regeneration of entire plants from somatic cells rather than through traditional sexual reproduction pathways. Additionally, transcription factors like WUSCHEL-RELATED HOMEOBOX (*WOX2*) are crucial for maintaining cellular totipotency and regulating the developmental pathways of somatic embryo. The totipotency of somatic plant cells is a specific and scientifically exciting phenomenon, which is based on the developmental program of plants. On the basis of study reported by Malabadi et al., (2012) [126] it is found that actively dividing and totipotent cells (stem cells) are positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division under in vitro conditions leading to a continuous flow of progeny cells [126]. These progeny cells (stem cells) under stress conditions (cold/heat) undergo differentiation due to signal activation in cambial region and leading to the embryogenic pathway in conifers [126]. On the other hand, the rest of the layers (epidermis, cortex region and central pith or medulla) of the transverse thin section of shoot apical meristems of mature trees have induced non-embryogenic tissue under in vitro conditions in conifers. The buds collected immediately after the bud burst were found very responsive for the *in vitro* cloning of *P. kesiya*, *P. roxburghii* and *P. wallichiana, P. patula*, and *P. sylvestris* [124]. This might be due the activation of the apical meristematic cells showing active growth of shoots in most of the conifers. The active dividing and totipotent cells might be positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division lead to a continuous flow of progeny cells [124].

According to the study conducted by Malabadi et al., (2012) [124] these cambial layer cells under stress conditions undergo differentiation and leading to the embryogenic pathway in conifers Under *in vitro* conditions, one or a few somatic cells of the plant or explant have to be competent to receive a signal (endogenous or exogenous). This then triggers the pathway of embryogenic development (commitment) leading to somatic embryo formation. *PrWOX2* (*Pinus roxburghii*) could be used as a potential genetic marker for the identification of the embryogenic cultures, which is just an added advantage for developing SE protocols for recalcitrant pines. Signal molecules regulating embryo development have been described in angiosperms, but very little is known about somatic rejuvenation in conifers. Recent studies on cloning of mature conifers provide new perspectives on signal molecules on cellular dedifferentiation into the embryogenic pathway. The identification of signal molecules such as butanolide, calcium ions, salicylic acid, antioxidants, amino acids, triacontanol and 24-epibrassinolide all play an important role in the conversion of somatic cells into an embryogenic pathway in many recalcitrant pines. This constitutes a major breakthrough in forest biotechnology with many practical applications in clonal forestry.

In addition to this Government of India has recognized this outstanding contribution of Professor Pramod Tandon for the initiation, establishment and successful regeneration of pine tissue culture in India [180-184, 172, 186, 188, 189-192, 193, 185]. Professor Pramod Tandon is an Indian Plant Biotechnologist and academic. He is a former Professor of Botany and Vice-Chancellor of North-Eastern Hill University (NEHU), Shillong, Meghalaya, India and Chief Executive Officer of Biotech Park, Lucknow. UP, India. The **Government of India** has awarded **Professor Pramod Tandon** the fourth highest civilian honor **Padma Sri in 2009, f**or his outstanding contribution to science.

However, commercialization of pine tissue culture work in India is still facing problems and major bottleneck due to the many reasons. At this point of time, pine tissue culture work in India is dead and remained as the academic research of the experimental model and failed commercialization. Furthermore, the protocol also suffered due to low plantlet regeneration, pine tissue culture research is time consuming, very slow and high risk. There are many cell separation and quantification techniques are available. Therefore, for the future studies, separation and identification of reprogrammed plant cells leading to the embryogenic pathway under *in vitro* conditions should be done and the applications of Artificial intelligence (AI) approach could solve many of the current issues.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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