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Review Article

Induction of somatic embryogenesis in mature coniferous forest trees

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Somatic embryogenesis using apical meristematic tissues of mature trees of conifers has many practical applications in the commercial forestry. However, induction of somatic embryogenesis in conifers has many challenges. This review paper highlights the recent achievements made in the somatic embryogenesis of conifers particularly the mature tree cloning. Our recent study showed that the induction of somatic embryogenesis in many recalcitrant pines is influenced by shoot bud collection timing, thin cell layers of apical meristems, nutrient medium composition, cambial layer activation (stem cells), smoke-saturated water, triacontanol, and 24-epibrassinolide. Therefore, the exact molecular events underlying the conversion from differentiation of somatic to embryogenic cell fate needs further studies in order to understand the somatic embryogenesis in conifers.

Key words: cloning, commercial forestry, *in vitro*, India, mature trees, micropropagation, thin cell layers

Abbreviations: AGP, arabinogalacton protein; ASA, acetyl salicyclic acid; BR, brassinosteroid; DCR, Durzan and Gupta medium; DTT, dithiothreitol; GlcN, *N*-glucosamine; EGTAethylenglycol-bis-(B-aminoethyl ether)-*N*,*N*,*N*,*N*,-tetra acetic acid; GlcNAc N-acetyl-glucosamine; La^{3+}, lanthanum chloride; LCO, lipophilic chitin ligosaccharide; OG, oligo-galacturonide; MS, Murashige and Skoog medium; PEM, pro-embryogenic mass; PGR, plant growth regulator; SA, salicyclic acid; SE, Somatic embryogenesis; SSW, smoke-saturated water; TDZ, thidiazuron; tTCL, transverse thin cell layer; TRIA, triacontanol; WOX2, *WUSCHEL* homeobox 2, transcription factor.

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1. Introduction

Production of embryo like structures under *in vitro* conditions by the external growth hormones is termed as somatic embryogenesis (Konar and Nataraja 1965; Nataraja and Konar 1970; Steward 1984; Feher *et al.* 2003; Malabadi *et al.* 2011). The totipotency of somatic plant cells is a specific and it is a inherent property of the cell (Konar and Nataraja 1965; Steward 1984; Ikeda-Iwai *et al.* 2003; Namasivayam 2007; Malabadi *et al.* 2009a). 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 (Carles and Fletcher 2003; Feher *et al.* 2003; Feher *et al.* 2003; Feher 2006).

Mature trees can not be regenerated under normal standard *in vitro* conditions; alternative approaches include the application of an abiotic stress (temperature, chemical, osmotic) to alter phase change (von Aderkas and Bonga 2000; Karami and Saidi 2009; Poethig 1990). At present, embryogenic systems 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, 1999; Paques and Bercetche 1998; Bonga 1996, 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, 2007c 2007f, 2007g, 2007e; Malabadi *et al.* 2008a, 2008b, 2008c; Park *et al.* 2009; Malabadi and Teixeira da Silva 2011), and an embryogenic system could be used for genetic transformation studies. This review paper highlights the recent updates on somatic embryogenesis in mature conifers.

2. Plant somatic embryogenesis

There are many influencing molecules regulating somatic embryogenesis has been reported in several plant species viz., carrot (*Daucus carota*), alfalfa (*Medicago sativa*), maize (*Zea mays*), Norway spruce (*Picea abies*) and chicory (*Cichorium*). In carrot, it was shown that when non-embryogenic cultures were treated with growth medium conditioned by highly embryogenic cultures, the cultures became embryogenic (Hari 1980). Several components in the conditioned growth medium promote somatic embryogenesis. These components include chitinases (De Jong *et al.* 1992; Egertsdotter *et al.* 1993; Dyachok *et al.* 2002), and arabinogalactan proteins (AGPs; Kreuger and Van Holst, 1993, Egertsdotter and von Arnold, 1995; Chapman *et al.* 2000). An endochitinase secreted into culture media was capable of rescuing a temperature-sensitive embryo-genesis defective mutant (De Jong *et al.* 1992). The signals are effective across species: a sugar beet (*Beta vulgaris*) endochitinase can stimulate early development of *P. abies* somatic embryos (Egertsdotter and von Arnold 1995, 1998). A basic chitinase secreted by *Pinus caribaea* embryogenic tissue, but not by non-embryogenic tissue, acts upon arabinogalacton protein (AGPs) from embryogenic tissue but not AGPs from non-embryogenic tissue (Dyachok *et al.* 2002; Cairney and Pullman 2007). AGPs,

glycosylated polypeptides consisting of up to 90% carbohydrate, are widely distributed, are commonly found in tissue culture, and are capable of stimulating somatic embryogenesis when added to a weakly embryogenic cell line (Kreuger and van Holst 1993; Dyachok *et al.* 2002; Cairney and Pullman 2007). AGP altered the morphogenetic response (root formation instead of embryogenesis) in carrot (Thompson and Knox 1998) and blocked somatic embryo formation in chicory (Chapman *et al.* 2000; Dyachok *et al.* 2002). Promotory and inhibitory effects of certain exogenous arabinogalactan protein fractions in carrot cultures were reported by Toonen *et al.* (1997) and later widely reviewed and discussed by Feher *et al.* (2003). It has been suggested that oligosaccharides released from AGPs by a chitinase act as signal molecules stimulating somatic embryogenesis (Van Hengel *et al.* 2001; Dyachok *et al.* 2002). It can be hypothesized that chitinase-modified AGPs are extracellular molecules capable of controlling or maintaining the embryogenic competent cell state (van Hengel *et al.* 2001; Dyachok *et al.* 2002). The relationship among AGPs, chitinases and lipophilic chitin oligosaccharides (LCOs) and their manner of stimulating embryogenesis has been well documented (Van Hengel *et al.* 2001; Dyachok *et al.* 2002; Wiweger, 2003; Feher *et al.*, 2003). AGPs isolated from immature seeds had an increased capacity to promote embryogenesis when pretreated with chitinase EP3 or AGP3 were extracted from endosperm (van Hengel *et al.* 2001; Dyachok *et al.* 2002). Therefore, AGP-mediated control of embryogenesis may be regulated by differential gene expression, and/or differential processing of the AGP-polypeptide moiety. AGPs and LCO as well as chitinases (EP3 and CH4) can stimulate somatic embryogenesis in Norway spruce (Daychok *et al.* 2002) and in carrot (De Jong *et al.* 1992). It has been suggested that LCOs are parts of AGPs that are released by chitinases. van Hengel and coworkers (2001) showed that LCO-like molecules are released from AGPs after they are hydrolysed by chitinases. Dyachok *et al.* (2002) reported that the endogenous LCO acts as a signal molecule stimulating pro-embryogenic masses (PEMs), and early embryo development in Norway spruce. Embryogenic cultures of Norway spruce are composed of PEMs and somatic embryos of various developmental stages. Auxin is important for PEM formation and proliferation (Dyachok *et al.* 2002). Depletion of auxin blocks PEM development and causes large-scale cell death in *P. abies* (Arnold *et al.* 2002). Extracts of the media conditioned by embryogenic cultures stimulate development of PEM aggregates in auxin-deficient cultures (Dyachok *et al.* 2002). Partial characterization of the conditioning factor shows that it is a lipophilic, low-molecular-weight molecule, which is sensitive to chitinase and contains GlcNAc (oligosaccharide backbone β-1,4-linked *N*-acetylglucosamine) residues (Dyachok *et al.* 2002). On the basis of this information, Dyachok *et al.* (2002) proposed that the factor is an LCO. The amount of LCO correlates to the developmental stages of PEMs and embryos, with the highest level in the media conditioned by developmentally blocked cultures. LCO is not present in non-embryogenic cultures of *Picea abies* (Dyachok *et al.* 2002; Wiweger, 2003). Cell death, induced by withdrawal of auxin, is suppressed by an extra supply of

endogenous LCO or Nod factor from *Rhizobium* sp. NGR234. The effect can be mimicked by a chitotetraose or chitinase from *Streptomyces griseus* (Dyachok *et al.* 2002; Wiweger, 2003). Nodulation (Nod) factors are produced by bacteria belonging to the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* in response to plant flavonoids (Spaink, 1996; Dyachok *et al.* 2002; Wiweger, 2003). Different rhizobia produce different sets of Nod factors with specific modifications, and these appear to determine host specificity (Staehelin *et al.* 1994b; Dyachok *et al.* 2002; Wiweger, 2003). However, Nod factors uniformly consist of an oligosaccharide backbone of GlcNAc tri-, tetra- or pentasaccharide, with an N-linked fatty acid moiety replacing the N-acetyl group on the non-reducing end (Wiweger, 2003). The length of the oligosaccharide chain, the acetylation at the non-reducing end and the sulfation at the reducing end of the LCO, influence the stability of the molecule against degradation by chitinases (Staehelin *et al.* 1994b; De Jong *et al.* 1992; Wiweger, 2003). Nod factors are known to induce cell divisions in the root cortex of the host legume, leading to the formation of nodules (Schultze and Kondorosi, 1996; Spaink, 1996; Wiweger, 2003). Rhizobial LCOs and chitin oligosaccharides stimulate the earliest stages of nodulation probably by perturbing the auxin flow in the root, and this auxin transport inhibition is probably mediated by endogenous flavonoids (Mathesius *et al.* 1998; Wiweger, 2003).

A number of studies have shown that Nod factors influence embryo development of non-leguminous plants (De Jong *et al.* 1992; Dyachok *et al.* 2000; Egertsdotter and von Arnold, 1995; Wiweger, 2003). Baldan and coworkers (2003) described the oligo-galacturonide (OG)-induced changes in the developmental pattern of somatic embryos in carrot (Wiweger, 2003). The response to OGs was strictly dependent on the developmental stage of the treated embryos (Baldan *et al.* 2003; Wiweger, 2003). Treatment of embryos at the globular stage resulted in the inhibition of the elongation of the axis and the formation of a multiple shoot apex (Wiweger, 2003). This is another example where treatment of embryos at early stages of development results in severe abnormalities during later development (Wiweger, 2003; Dyachok *et al.* 2002; Wiweger, 2003).

It has long been known that Nod factors produced by rhizobia induce cell divisions in the root cortex of the host legume, leading to the formation of nodules (Truchet *et al.* 1991; Wiweger, 2003). Furthermore, in Norway spruce, Nod factors can substitute for auxin and cytokinin to promote cell division (Dyachok *et al.* 2002; Wiweger, 2003). In embryogenic maize cells, the extracellular matrix surface network has also been shown to contain arabinogalactan proteins (AGPs), as well as the antibody JIM4 arabinogalactan protein epitope, that were not present on the surface of non-embryogenic cells (Samaj *et al.* 1999; Wiweger, 2003; Namasivayam *et al.* 2010). In embryogenic cultures of *Cichorium*, the major secreted proteins were also identified as chitinases, glucanases and an osmotin-like protein, all of which accumulated at a higher level in embryogenic cultures compared to non-embryogenic cultures (Helleboid *et al.* 2000b). Similarly, in embryogenic alfalfa (*Medicago sativa*) cultures, alterations in the levels of extracellular proteins homologous to the carrot endochitinases have been

reported following the removal of 2,4-dichlorophenoxy acetic acid (2,4-D) (Poulsen *et al.* 1996; Feher *et al.* 2003).

Auxin and cytokinins are the main PGRs in plants involved in the regulation of cell division and differentiation.An endogenous auxin pulse is one of the first signals leading to the induction of somatic embryogenesis (Thomas *et al.* 2002; Wiweger, 2003). The influence of exogenously applied auxins, particularly 2,4-D, on the induction of somatic embryogenesis using vegetative shoot buds and secondary needles of mature pines, embryo cloning and mature zygotic embryos are well documented (Malabadi *et al.* 2004; Malabadi and van Staden, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007; Malabadi and Nataraja, 2007f, 2007h; Malabadi *et al.* 2008a, 2008b, 2008c).

Endogenous PGR levels, however, can be considered as major factors in determining the specificity of cellular responses to these rather general stress stimuli. In addition to the absolute requirement of exogenous auxins for sustained growth in *in vitro* cultures, plant cells may produce substantial amounts of the native auxin, indole-3-acetic acid (IAA) (Feher *et al.* 2003). Higher endogenous IAA concentrations are associated with increased embryogenic response in various plant species such as wheat, maize and carrot or their explants (Jimenez and Bangerth, 2001a, 2001b, 2001c). Among the different auxin analogues used to induce somatic embryogenesis, 2,4-D is by far the most efficient and, therefore, this synthetic PGR is used in the majority of embryogenic cell and tissue culture systems (Feher *et al.* 2003). 2,4-D above a certain concentration has a dual effect in these cultures, as an auxin (directly or through endogenous IAA metabolism) and as a stressor (Feher *et al.* 2003). 2,4-D is an auxinic herbicide with diverse effects associated with its phytotoxic activity, which cannot be ascribed simply to an auxin overdose (Feher *et al.* 2003). Auxinic herbicides have been shown to interact with ethylene and abscisic acid (ABA) synthesis, increasing the cellular levels of these called stress hormones (Feher *et al.* 2003). During cloning of mature pine trees, a combination of α-naphthalene acetic acid (NAA), 2,4-D and 6-benzyl adenine (BA) at particular concentrations induced embryogenic tissue on DCR (Gupta and Durzan, 1985) induction medium. The optimum concentration the external PGRs was 22.62 µM 2,4-D, 26.85 µM NAA and 8.87 µM BA (Malabadi *et al.* 2004), and transverse thin cell layers (tTCL), the ideal explants, induced embryogenic tissue in many pines such as *Pinus kesiya*, *P. roxburghii*, *P. wallichiana*, *P. patula*, *P. sylvestris*, *P. pinea* and *P. pinaster* (Malabadi *et al.* 2004; Malabadi and van Staden 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007; Malabadi and Nataraja, 2007f, 2007h; Malabadi *et al.* 2008a, 2008b, 2008c). Higher or lower concentrations of NAA, 2,4-D and BA resulted in the induction of non-embryogenic tissue. Interestingly, cell cultures of different ages had different sensitivities to cytokinin treatment. In pines, the formation of somatic embryos is influenced by the application of ABA in the maturation medium, without PGRs (Malabadi and van Staden, 2003, 2005a, 2005b, 2005c). This finding suggests that, as a stress signal, exogenous ABA is effective only in the absence of external PGRs (Feher *et al.* 2003). Therefore, the dedifferentiation of

a somatic cell is influenced by many factors such as stress conditions, and in such a case, simultaneous activation of auxin and stress responses may be key events in cellular adaptation, causing genetic, metabolic and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells (Feher *et al.* 2003).

3. Cloning of mature conifers

In vitro somatic embryogenesis is associated with artificial conditions, high levels of exogenous PGRs and many other stress factors. These extreme and stressful conditions may result in a general stress response in cells showing activity of signaling molecules involved in the conversion of somatic cells isolated from apical meristematic tissues (i.e., young tissue) of mature conifers (Feher *et al.* 2003; Feher, 2006; Karami and Saidi, 2009). Therefore, not all cells of an explant subjected to the same treatment are capable of developing into somatic embryos, explaining why various explants, genotypes and species need different conditions for successful induction of embryogenic tissue in conifers.

In the following sections the most important influencing molecules that play an important role in the conversion of somatic cells isolated from the shoot apical meristem of many recalcitrant mature conifers are described.

A. Calcium

In plants, Ca^{2+} functions as a second messenger in the signal transduction of a variety of environmental stimuli, which regulate and mediate plant embryogenesis (Luo *et al.* 2003; Jansen *et al.* 1990; Poovaiah and Reddy, 1993). The elevated Ca^{2+} concentration counteracted the inhibitory effect of 2,4-D on embryo development (Jansen *et al.* 1990; Feher *et al.* 2003; Luo *et al.* 2003). In carrot, somatic embryogenesis activated calmodulin (CaM) localized to regions undergoing rapid cell division, and an increase in the level of CaM mRNA was observed during globular and heart-shaped stages (Overvoorde and Grimes, 1994; Feher *et al.* 2003). This could be blocked by chelating exogenous Ca^{2+}, which arrested somatic embryo development, but still allowed cell proliferation to continue (Feher *et al.* 2003;Anil and Rao, 2000; Feher *et al.* 2003). Experiments with calcium channel blockers calcium chelators used in somatic embryogenesis of sandalwood (*Santalam album*) and carrot indicated that the influx of exogenous calcium is essential for the initiation of somatic embryogenesis (Overvoorde and Grimes, 1994; Anil and Rao, 2000; Luo *et al.* 2003). Increasing evidence has demonstrated that the elevation of cytoplasmic-free calcium ($(Ca^{2+})_{cyt}$) plays an important role in the response of plants to cold pretreatment shock (Malabadi and van Staden, 2006). In an embryogenic carrot cell suspension, an upward shift in the exogenous Ca^{2+} concentration (from 10^{-3} to 10^{-2} M) at the time of transfer to auxin-free embryo differentiation medium increased the number of somatic embryos approximately two-fold (Jansen *et al.* 1990; Feher *et al.*, 2003).

A study conducted in *Pinus patula* showed the necessity of a Ca^{2+} pool for embryogenesis (Malabadi and van Staden, 2006). Exogenous Ca^{2+} influenced somatic embryogenesis using vegetative shoot apices of mature trees in three genotypes. The

percentage of somatic embryogenesis in *P. patula* was significantly affected by increasing the exogenous Ca^{2+} concentration from 0 to 4 mM on the cold pre-treatment medium (I) and during the establishment of embryogenic suspension cultures. The highest percentage of explants showing embryogenesis (PP3: 8%, PP13: 10%, PP18: 3.5%; PP3 = *P. patula* no. 3; PP13 = *P. patula* no. 13; PP18 = *P. patula* no. 18) was recorded for the three *P. patula* genotypes after the addition of 4 mM of exogenous Ca^{2+} during the cold pretreatment or during cell suspension culture when compared to controls (0 mM Ca^{2+}) (PP3: 4.5%, PP13: 3.7%, PP18: 2.0%). The incorporation of exogenous Ca^{2+} at 2 mM during cold pretreatment at 2°C and during the establishment of embryogenic cell suspension cultures did not have a marked effect on embryogenesis in *P. patula* but an increase in the exogenous Ca^{2+} concentration to 5 mM significantly reduced the percentage of somatic embryogenesis following cold pretreatment and during establishment of cell suspension cultures. Therefore, 4 mM of exogenous Ca^{2+} was the optimum concentration for embryogenesis in *P. patula*. Hence, exogenous Ca^{2+} at 4 mM in DCR basal medium during cold pretreatment supported the expression of cold-enhanced capacity for somatic embryogenesis in *P. patula*. Furthermore, when the residual Ca^{2+} in the cold pretreatment medium was chelated with 2 mM ethyleneglycol-bis-(B-aminoethyl ether)-*N*,*N*,*N*,*N*-tetra acetic acid (EGTA), somatic embryogenesis in *P. patula* was significantly inhibited in pretreated suspension cultures compared with the control. These results suggest that cold-enhanced embryogenesis was related to Ca^{2+} in the medium used for cold pretreatment and that an optimum concentration (4 mM) was required during cold pretreatment for the expression of cold-enhanced embryogenesis in *P. patula* (Malabadi and van Staden, 2006). Additional data support that Ca^{2+}-dependent protein kinases (CDPKs) are involved in the signaling pathways during the formation of somatic embryos in sandal wood (Anil and Rao, 2000).

B. Salicyclic acid

There are many diverse physiological processes, due to the application of salicyclic acid has been reported in plants (Raskin *et al.* 1987; Raskin, 1992; Dean and Delaney, 2008). On the other hand, there are several reports describing exogenous SA and acetylsalicyclic acid (ASA) enhancing somatic embryogenesis in plants viz. carrot (Roustan *et al.* 1990), pearl millet (*Pennisetum americanum*) (Pius *et al.* 1993), geranium (Hutchinson and Saxena, 1996), *Astragalus adsurgens* Pall (Luo *et al.* 2001), *Plumbago rosea* L. (Komaraiah *et al.* 2004), naked oat (*Avena nuda*) (Hao *et al.* 2006) and *Pinus roxburghii* (Malabadi *et al.* 2008a, 2008b).

In a study of mature cloned *P. roxburghii* trees, pre-treatment (5 min) of shoot-tip tTCL explants of 10 different Chir pine genotypes with different concentrations of SA did not induce embryogenic tissue any more than the control (Malabadi *et al.* 2008a, 2008b). All genotypes showed a mixed response in embryogenic tissue induction following pretreatment of tTCLs with SA (in general). The pre-treatment of tTCLs from any of the 10 genotypes with 0.1, 0.2 and 0.4 mg^{-1} SA could not effectively increase the percentage of somatic embryogenesis when compared to the control. Two genotypes of *P. roxburghii*, PR-05 and PR-92, failed to induce

embryo-genic tissue following pre-treatment of explants with any concentration of SA, i.e., these two genotypes were completely recalcitrant to this treatment. Pretreatment of explants with higher concentrations (2.0-5.0 mg^{-1}) of SA might have had a toxic effect and resulted in the browning of explants without callus formation in all 10 genotypes of *P. roxburghii*. The percentage of responsive explants that could induce embryogenic tissue increased (significantly in some cases) from 7 to 12% in PR-811, 3 to 5% in PR-32, 6 to 8% in PR-805, and 11 to 16% in PR-821 following the pre-treatment of 1.0 mg^{-1} SA when compared with the control in *P. roxburghii*. This trend was also similar with 0.5 mg^{-1} SA where the percentage of responsive based explants for inducing embryogenic tissue increased from 7 to 9% in PR-811 and 11 to 14% in PR-821, respectively. Therefore, pre-treatment with 0.5 or 1.0 mg^{-1} SA was optimum at least in a few *P. roxburghii* genotypes (PR-811, PR-32, PR-805, PR-821) for improving the percentage of somatic embryogenesis. Incorporation of 1.0 mg^{-1} SA in the induction medium was optimum for all 10 genotypes by increasing the percentage of somatic embryogenesis compared to the control. The highest percentage (31%) of somatic embryogenesis was recorded in PR-821 and PR-46. For PR-05, in particular, the addition of 1.0 mg^{-1} SA to the induction medium was very beneficial since in the control this genotype failed to induce somatic embryogenesis. This clearly indicates the positive role of SA as a signaling molecule during cloning of mature *P. roxburghii* trees. In these studies, SA alone (i.e. without PGRs) did not induce somatic embryogenesis and resulted in the browning of explants and callus. Microscopic observation showed simple, elongated parenchymatous cells without any sign of cleavage polyembryony. SA, when combined with 22.6 μM 2,4-D, 26.8 μM NAA and 8.9 μM BA in the induction medium improved the percentage of somatic embryogenesis in *P. roxburghii*. However, in PR-05, this synergistic mix induced only 3% somatic embryogenesis while in PR-05 it failed to induce somatic embryogenesis. Hence, the combination of SA with other PGRs such as 2,4-D/NAA/BA might be beneficial in inducing somatic embryogenesis in Chir pine (Malabadi *et al.* 2008a, 2008b). In geranium, thidiazuron (TDZ) effectively induced somatic embryogenesis in cultured hypocotyls explants during only a 3-day period of induction (Hutchinson and Saxena, 1996). However, in the same study, SA was ineffective in modulating similar embryo-genic responses as ASA in geranium (Hutchinson and Saxena, 1996). Enhanced somatic embryogenesis and plant regeneration have been obtained using young leaf bases of naked oat as explants by including 0.5 mM SA and carrot embryogenic callus extracts in MS media. Somatic embryogenesis and plant regeneration were improved on the corresponding media supplemented with 0.5 mM SA and carrot embryogenic callus extracts compared to the control (Hao *et al.* 2006). Somatic embryo-genesis was induced from suspension cultures derived from leaf callus of an important medicinal plant, *Plumbago rosea* L. (Komaraiah *et al.* 2004) in which 8.32 μM ASA alone induced embryogenesis, but IAA, NAA or IBA alone failed to elicit a similar response. Optimal embryogenic response per culture (216 embryos/culture) was observed in MS

medium containing a combination of ASA (8.32 μM) and IAA (5.06 μM), i.e. a similar synergistic response as observed in our study between SA/ASA and other PGRs in the medium. It was also observed that by increasing the concentration of ASA alone (without auxin) in the medium (up to 11.09 μM) the number of somatic embryos formed per culture increased (Komaraiah *et al.* 2004). The interactive effect of ASA and IAA appears to be essential for enhanced production of embryos per culture since no embryogenesis was noticed when IAA alone was added in *P. rosea* (Komaraiah *et al.* 2004). SA is endogenously produced in many plants in normal and stressed conditions (Raskin *et al.* 1987). Therefore, one possible link between oxidative stress and plant regeneration in tissue culture could be hydrogen peroxide, or H_2O_2. SA is endogenously produced in many plants in normal as well as during stress conditions (Raskin *et al.* 1987). SA also inhibited ethylene biosynthesis in cell suspension cultures of carrot (Roustan *et al.* 1990). Ethylene inhibits differentiation in plants. During the study of cloning of mature trees of *P. roxburghii*, SA may be promoting embryo development by inhibiting ethylene biosynthesis (Malabadi *et al.* 2008a, 2008b). Another hypothesis is that SA has been reported to increase the activity of superoxide dismutase (Rao *et al.* 1997), and inhibits the activities of ascorbate peroxidase and catalases, thus leading to endogenous H_2O_2 accumulation in *Arabidopsis thaliana* (Rao *et al.* 1997).

C. Triacontinol

The plant growth-stimulating property of TRIA was demonstrated for the first time by Crosby and Vlitos (1959) and later by Stoutemyer (1981), Stoutemyer and Cooke (1987) and Tantos and coworkers (1999, 2001). TRIA, a long 30-carbon primary alcohol, is a naturally occurring plant growth promoter (Ries *et al.* 1977; Ries and Houtz, 1983; Ries and Wert, 1982, 1988) and a component of the epicutcular waxes of alfalfa and many other plants (Chibnall *et al.* 1933; Azam *et al.* 1997). TRIA induced somatic embryogenesis in *Pinus roxburghii* and *P. kesiya* has been reported for the first time in conifers (Malabadi and Nataraja, 2005, 2007c). During that study, mature zygotic embryos cultured on full-strength LM (Litvay *et al.*, 1985) basal medium supplemented with 9.0 jiM 2,4-D and lower concentrations of TRIA (4, 5 and 7 jig 1^{-1}) induced white mucilaginous embryo-genic callus in all three genotypes (Malabadi and Nataraja, 2005, 2007c). Mature zygotic embryos induced a higher percentage of white glossy non-embryogenic callus with lower (1, 2 jig1^{-1}) and higher concentrations of TRIA (15, 20, 25, 30 jig 1^{-1}) on full-strength LM basal medium in all three genotypes. The most effective range of TRIA which induced white-mucilaginous embryogenic callus on full-strength LM basal medium containing 9.0 jiM 2,4-D was 4-7 jig $^{1-1}$ in all three genotypes. The highest percentage of white mucilaginous embryogenic callus was induced on full-strength LM basal medium supplemented with 9.0 jiM 2,4-D and 7 jig 1^{-1} TRIA (initiation medium I) (Malabadi and Nataraja, 2007c). The positive effect of TRIA as a growth regulator was studied by various groups on herbaceous and woody plant micro-propagation (Tantos *et al.* 1999; Fraternale *et al.* 2002, 2003). According to Reddy *et al.* (2002), an average of 2-3 shoots with an increase in the number of nodes and

leaves were noticed in *Capsicum frutescens* and *Decalepis hamiltonii* after 5 jig l^{-1} TRIA treatment formed longest shoots in the medium. TRIA also promoted shoot multiplication in *Melissa officinalis* (Tantos *et al.* 1999). A lower concentration of TRIA may be biologically effective because of the sensitivity of whole explants to extremely low doses of TRIA (Biernbaum *et al.* 1998; Ries and Houtz, 1983) and growth processes (Ries *et al.* 1977). In another recent study, an efficient somatic embryogenesis protocol was developed for the first time using TRIA as a PGR in *Catharanthus roseus* (Malabadi *et al.*, 2009b). In this study, friable embryogenic callus was induced from thin sections of shoot tips collected from field-grown plants on MS medium supplemented with 2.0 jiM 2, 4-D and 5.0 jiM TRIA (induction medium I) (Malabadi *et al.*, 2009b).

D. Smoke

The ability of plant-derived smoke to break dormancy and stimulate germination was first reported for *Audouinia capitata*, a fynbos species growing in a fire-prone habitat (Light and van Staden, 2004). Smoke is an important factor involved in fire and post-fire germination cues (van Staden *et al.* 2000; Jain and van Staden, 2006; Jain *et al.* 2008). Smoke from the combustion of plant material stimulates seed germination in a wide range of species (Light and van Staden, 2004; Malabadi and Vijaya Kumar, 2006, 2007b; Daws *et al.* 2008). A highly active germination promoting compound has recently been identified as a water-soluble butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one, from the smoke of burnt fynbos *Passerina vulgaris* Thoday and the grass *Themeda triandra* L. (van Staden *et al.* 2004) as well as from the combustion of cellulose (Flematti *et al.* 2004). The recent identification of the germination cue, butenolide from smoke will now allow for research into the physiological action of smoke on seed germination. Malabadi *et al.* (2008c) reported the influence of smoke-saturated water (SSW) on asymbiotic seed germination and an early differentiation of protocorms and plant regeneration of *Vanda parviflora* Lindl. SSW does not have any significant effect on the germination period of somatic embryos in all the three genotypes of *P. wallichiana* although it did affect the total number of somatic embryos that germinated (Malabadi and Nataraja, 2007a; Malabadi *et al.*, 2009). In geranium (*Pelargonium hortorum* Bailey cv. 'Elite'), SSW treatment (10% v/v) of the explant prior to induction, or together with the inductive signal (TDZ) produced the highest number of somatic embryos (Senaratna *et al.* 1999; Malabadi *et al.*, 2009).

In another study, the effect of butenolide, 3-methyl-2*H*-furo [2, 3-c] pyran-2-one was tested for its effect on somatic embryogenesis with an important species for commercial horticulture, *Baloskion tetraphyllum* (Restionaceae) (Ma *et al.* 2006). It was observed that when somatic embryos of *B. tetraphyllum* were transferred to basal medium (MS) supplemented with 0.067 jiM butenolide, the development of growth-competent somatic embryos was enhanced using different explants such as shoots and coleoptiles (Ma *et al.* 2006). Butenolide resulted in a high frequency of somatic embryos progressing to plantlets, and a higher number of plantlets per explant compared to non-butenolide (control) media for both shoot and coleoptile explants in *B.*

tetraphyllum (Ma *et al.* 2006). The number of somatic embryos doubled following the addition of SSW at either the explant or induction stage compared to the untreated control in *P. wallichiana* (Malabadi and Nataraja, 2007a; Malabadi *et al.*, 2009c). The inductive signals for the initiation of somatic embryogenesis of *P. wallichiana* were BA, NAA and 2,4-D. SSW without BA, NAA or 2,4-D did not induce any form of cell proliferation; however, SSW appeared to act synergistically with the inductive signal (Malabadi and Nataraja, 2007a; Malabadi *et al.*, 2009c). Therefore, SSW has a stimulatory role during cloning of mature trees of *P. wallichiana*, and thus stimulates the conversion of somatic cell into an embryogenic pathway (Malabadi and Nataraja, 2007a; Malabadi *et al.*, 2009c).

E. 24-Epibrassinolide

Brassinosteroids (BRs) are a group of naturally occurring steroidal lactones that include brassinolide and its analogs. In several bioassays, they have been reported to affect cell elongation, division and differentiation of plant cells (Grove *et al.* 1979; Mandava, 1988; Sakurai and Fuijoka, 1993, 1997; Mayumi and Shibaoka, 1995; Clouse *et al.* 1996; Clouse and Sasse, 1997; Fujioka *et al.* 1998; Fujioka, 1999; Altmann, 1999; Khripach *et al.* 2000; Ozdemir *et al.* 2004). Others reported the initiation of embryogenic tissue in conifers, cotton, organogenesis in sweet pepper and cauliflower on media using 24-epibrassinolide or 24-epiBR (Pullman *et al.* 2003; Wang *et al.* 1992; Franck-Duchenne *et al.* 1998; Sasaki, 2002). In various bioassays, brassinolide has been shown to be more active than, or synergistic with, auxins such as IAA or NAA (Brosa, 1999). Work with Chinese cabbage (*Brassica rapa*) protoplasts has shown that 24-epiBR promoted cell division in the presence of 2,4-D and kinetin (Nakajima *et al.* 1996). Oh and Clouse (1998) demonstrated that brassinolide increased the rate of cell division in isolated leaf protoplasts of *Petunia hybrida*. Ronsch *et al.* (1993) reported an improvement in the rooting efficiency and survival of Norway spruce seedlings using 22S, 23S-homo-brassinolide. Embryogenic callus induction and growth of coffee, lettuce and potato was improved by the use of spirostane analogues of BRs in the culture medium as a cytokinin substitute or complement (Lu *et al.* 2003; Oh and Clouse, 1998; Nakajima *et al.* 1996; Nunez *et al.* 2004). Hu *et al.* (2000) suggested that 24-epiBR may promote cell division through Cyc D3, a D-type plant cyclin gene through which cytokinin activates cell division. They also showed that 24-epiBR can substitute cytokinin in culturing *Arabidopsis* callus and suspension cells.

More recently, in conifers, mature zygotic embryos produced white mucilaginous embryogenic tissue on MSG containing 9.0 µM 2,4-D and 24-epiBR at 0.5, 1.0 and 2.0 µM (Malabadi and Nataraja, 2007d). Mature zygotic embryos produced the highest percentage of embryogenic tissue on half-strength MSG (Becwar *et al.* 1990) medium supplemented with 9.0 µM 2,4-D and 2.0 µM 24-epiBR (initiation medium) in all three genotypes of *P. wallichiana* tested. The highest percentage of somatic embryogenesis (91.5 ± 3.0a) was recorded in genotype PW106. On the other hand, the lowest percentage of somatic embryogenesis (60.8 ± 4.0 b) was obtained in genotype PW21 (Malabadi and Nataraja, 2007d). Although,

little information is available for conifers, BRs have been isolated from conifers (Kim *et al.* 1990) and exogenous applications of BRs to pine seedlings and spruce cuttings have shown improved root growth, whole plant growth, or both (Ronsch *et al.* 1993; Rajasekaran and Blake, 1998). Pullman *et al.* (2003) reported that use of brassinolide at 0.1 µM improved the percentage of embryogenic cultures in loblolly pine (*Pinus taeda*), Douglas fir (*Pseudotsuga menziesii*), and Norway spruce. They also showed that brassinolide increased the weight of loblolly pine embryogenic tissue by 66% and stimulated initiation of embryogenic tissue in the more recalcitrant families of loblolly pine and Douglas fir, thus compensating somewhat for genotypic differences in initiation (Pullman *et al.* 2003). BRs are of ubiquitous occurrence in plants and elicit a wide spectrum of physiological responses (Gupta *et al.* 2004). In angiosperms, BRs have several effects, including stimulating cell division, ethylene production, and adventitious tissue formation and increasing resistance to abiotic stress (Brosa, 1999). Two spirostane analogues of BRs (BB6 and MH5) were tested for callus induction and plant regeneration in lettuce (*Lactuca sativa*). Results indicated that both BB6 and MH5 enhanced both callus formation and shoot regeneration from cotyledons in lettuce (Nunez *et al.* 2004). In the case of rice seeds, the application of BRs reduced the impact of salt stress on growth, prevented photosynthesis pigment loss and increased nitrate reductase activity (Anuradha and Rao, 2003). Embryogenic callus induction and growth of coffee (*Coffea arabica*) and potato (*Solanum tuberosum*) was improved by the use of spirostane analogues of BRs in the culture medium as a cytokinin substitute or complement (García, 2000; More *et al.* 2001). Frank-Duchenne *et al.* (1998) used 0.1 µM 24- epiBR to enhance the elongation of shoots grown through direct organogenesis on cotyledons and hypocotyls with a regeneration-recalcitrant cultivar of sweet pepper (*Capsicum annuum*). Sasaki (2002) used brassinolide to increase adventitious shoot production on cauliflower (*Brassica oleracea*) hypocotyls segments. Wang *et al.* (1992) obtained embryogenic cotton (*Gossypium herbacium*) cultures from seedling hypocotyls with the aid of 0.02 µM brassinolide. These results indicated ample evidence that BRs possess a broad spectrum of biological activities compared to the known plant PGRs, including gibberellin, auxin and cytokinin-like activities (Yopp *et al.* 1981; Brosa, 1999).

F. Molecular evidence of SE regulation

Re-programing and gene expression plays an important role in somatic embryogenesis of plants (Chugh and Khurana, 2002; Namasivayam, 2007). Chugh and Khurana, 2002; Namasivayam, 2007). The gene expression have been extensively studied in carrot, alfalfa and chicory (Namasivayam, 2007). There is only one gene known to play a role in the acquisition of embryogenic competence in plant cells. This is the somatic embryogenesis receptor kinase (*SERK*) gene isolated by Schmidt *et al.* (1997). In carrot, *SERK* expression was shown to be characteristic of embryogenic cell cultures and somatic embryos, but its expression ceased after the globular stage (Schmidt *et al.* 1997; Namasivayam, 2007; Schmidt *et al.* 1997; Chugh and Khurana, 2002). The expression of a *SERK* homologue gene was also tested in *Dactylis glomerata*, where

somatic embryogenesis could be initiated directly from leaf explants, as well as indirectly from leaf-derived callus tissues (Somleva *et al.* 2000; Feher *et al.* 2003). In contrast to carrot and *Arabidopsis*, *SERK* expression was maintained beyond the globular stages of embryogenesis in meristematic regions (Somleva *et al.* 2000; Namasivayam, 2007). Other leucine rich repeat-containing receptor-like kinases are known to be involved in different developmental processes in plants, such as the *ARABIDOPSIS CLAVATA* and *ERECTA* proteins (for review, see Fletcher and Meyerowitz, 2000) and the *Petunia PRK1* protein, which is involved in signaling during pollen development and pollination (Mu *et al.* 1994; Feher *et al.* 2003). Recently, a cDNA of another MADS-box containing transcription factor was identified by investigating differentially expressed genes during *Brassica* microspore embryogenesis (Boutilier *et al.* 2000, 2002; Feher *et al.* 2003; Namasivayam, 2007). When this cDNA was overexpressed under the control of the 35S promoter in transgenic plants, ectopic formation of embryos and cotyledons on leaves was observed, due to which the gene was named *BABYBOOM* (*bbm*) (Boutilier *et al.* 2000, 2002; Chugh and Khurana, 2002; Feher *et al.* 2003; Namasivayam, 2007).

WUS was originally identified as a central regulator of shoot and floral meristems in *Arabidopsis* (Mayer *et al.* 1998) where it is expressed in a small group of cells, and is required to maintain the overlying stem cells undifferentiated (Arroyo-Herrera *et al.* 2008; Zuo *et al.* 2002; Arroyo-Herrera *et al.* 2008). In *Arabidopsis*, *WUS* has been found to be sufficient to ectopically induce stem cells (Zuo *et al.* 2002; Arroyo-Herrera *et al.* 2008). Arroyo-Herrera *et al.* (2008) found that expression of *WUS* in coffee plants can induce calli formation as well as a 400% increase somatic embryo production. However, a critical developmental stage and additional hormonal requirements are required for the induction of embryogenesis by *WUS* in *Coffea canephora* (Arroyo-Herrera *et al.* 2008; Karami *et al.* 2009; Zuo *et al.* 2002). Moreover, no callus phase, or at least only few cell-division cycles, is sufficient to induce cells to restart a totally new embryogenic pathway in tissues of plants that over-express *WUS* (Zuo *et al.* 2002; Trigiano and Gray, 2010).

Recently for the first time, the expression of cDNA clones of genes involved in programming the apical meristem cells towards somatic embryogenic pathway influenced by external environmental stimulus like cold – pretreatment has been reported in *P. roxburghii* (Malabadi and Nataraja, 2007g). Differential display was used to isolate the genes which are expressed specifically in embryogenic tissue induced by cold-pretreatment of thin sections of vegetative shoot apices of mature trees of *Pinus roxburghii*. Of the 56 cold-enhanced embryogenic-associated cDNAs identified, 20 were cloned (Malabadi and Nataraja 2007g). During reverse northern hybridization, all the 20 clones selected generated a positive signal when probed with labeled cDNA from cold-enhanced embryogenic tissue, but no signal when probed with cDNA from the non-embryogenic tissue (control treatment). All the 20 clones thus contained inserts that were specific to cold-enhanced somatic embryogenesis. The identification of genes

revealed the expression of *WOX2* transcription factor (*WUSCHEL homeobox 2*) (unpublished data) in the embryogenic tissue derived from the apical meristematic tissue from mature trees of *P. roxburghii* (Malabadi and co-workers-unpublished work). This clearly indicates the involvement of *WOX2* in the induction of embryogenic tissue from the apical meristem in *P. roxburghii*. Therefore, homeobox transcription factor *WOX2* might be involved in the molecular mechanism that mediates the vegetative-to-embryogenic transition during cloning of mature trees of *P. roxburghii* (Malabadi and co-workers-unpublished work). In another recent study, putative embryogenesis-specific genes, *WOX2* (*WUSCHEL* homeobox 2) and *HAP3A*, were analyzed in cultures of both shoot bud explants and ZEs (Park *et al.* 2009). The findings from this study, based on molecular assessment, suggest that the cell lines derived from bud cultures were truly EM (Park *et al.* 2009). Moreover, these experimental observations suggest that *PcWOX2* could be used as an early genetic marker to discriminate embryogenic cultures from callus (Park *et al*. 2009).

4. Recent updates on SE in conifers

A recent study showed a remarkable evidence of the involvement of cambial layer produced embryogenic cells. 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. This is one of the important steps when we work on cloning of mature conifers. 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. 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* conditions. 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.sylvistris,* and Lodgepole pine (Aronen *et al*. 2007, 2008; Aronen, 2009; Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007; Park *et al.* 2009). This might be due the activation of the apical meristematic cells showing active growth of shoots in most of the conifers.

Free radicals are frequently generated by wounding and may result in an increase in the activity of peroxidase and catalase enzymes, which act to overcome the effect of oxidizing radicals (Benson, 2000; Malabadi and van Staden, 2005b). Several antioxidants like cysteine, dithiothreitol and a combination of ascorbic acid (AA) with citric acid (CA) have been evaluated, with varying success, in the management of phenolic damage to excised tissue. Pretreatment of thin shoot apical dome sections or tTCLs of *Pinus patula* with different concentrations of antioxidants (CA, AA, cysteine, dithiothreitol (DTT) (Sigma) (0, 0.01, 0.05, 0.1%), CA/AA (Sigma) (0.01, 0.1, 0.5%) and tri-potassium citrate (Sigma) (KC) combined with CA in equal concentrations (0.01 0.1, 0.05% KC/CA), for 10 min, or including antioxidants directly in the DCR culture media, reduced somatic embryo production compared with controls pretreated with sterile distilled water only

(Malabadi and van Staden, 2005b). However, pretreatment of explants (tTCLs) with the antioxidants had a positive effect by reducing the degree of tissue necrosis and reducing the oxidation of phenolic compounds. The explants thus remained green without browning. However, the percentage of surviving cultures and somatic embryo production decreased when pretreated with antioxidants such as CA, AA, AA/CA and KC/CA respectively. In all three genotypes of *P. patula*, the untreated controls showed a higher percentage of embryogenic culture initiation than when pretreated with antioxidants. The exception being dithiothreitol (DTT) at 0.1% which increased the percentage of embryogenic cultures and degree of somatic embryo production. Higher concentrations of DTT decreased the percentage of embryogenic cultures and somatic embryo production during cloningof mature trees of *P. patula* (Malabadi and van Staden, 2005b).

The addition of almost all antioxidants to the DCR culture media or a pretreatment reduced production of somatic embryos during cloning of mature *P. patula* trees (Malabadi and van Staden, 2005b). In the present study, DTT had a positive effect on the initiation of embryogenic cultures at 0.1%. Presently it is not known how DTT influences embryogenesis. This simple procedure of preparing explant material using DTT (0.1%) prior to culture results in a reduction of tissue death caused by browning (phenolic oxidation), consequently enhancing initiation of embryogenesis of shoot apical domes of mature *P. patula* trees (Malabadi and van Staden, 2005b) and could thus solve the problem of browning in other conifer somatic embryogenesis protocols.

Somatic embryo production was promoted with the addition of amino acids in geranium (*Pelargonium* × *hortorum* Bailey) (Murthy *et al.* 1996). The effect of amino acids on embryo maturation and somatic seedling recovery in three genotypes (PP3, PP13, and PP18) of *Pinus patula* that exhibited a good response in terms of somatic embryo recovery (Malabadi and van Staden, 2005b). The highest percentage of somatic embryogenesis (19%) and somatic seedling recovery (15) was with genotype PP3 and was recorded with 10.0 mM of the amino acid mixture (Malabadi and van Staden, 2005b). Garin *et al.* (2000) reported that a combination of amino acids incorporated in the maturation medium either had no effect or decreased somatic embryo production of *Pinus strobus*, depending upon the embryogenic line. However, in *Picea glauca*, somatic embryos matured in the absence of glutamine, but in the presence of casein hydrolysate and inorganic nitrogen. Smith (1994) supplemented with amino acids in the maturation medium of *Pinus radiata* to improve somatic embryo production and plantlet recovery, with a solution of seven amino acids and reported significant effects on somatic embryogenesis (Malabadi and van Staden, 2005b). In other plant species, such as alfalfa and *Picea glauca* some amino acids had either a negative or no effect on somatic embryo maturation. During cloning of mature trees of *P. patula*, apart from dithiothreitol at 0.1%, pre-treatment of tTCL explants or incorporation of antioxidants in the DCR basal nutrient medium had a negative effect on the initiation of embryogenic cultures, somatic embryo production and plantlet recovery.

Competent cells can respond to a variety of conditions by the initiation of embryogenic development. It can also be hypothesized that the initiation of somatic embryogenesis using mature conifers is a general response to a multitude of parallel signals (including growth regulators, stress factors, physiological status of buds, carbon source, calcium ions, and alteration of gene expression). Recently cloning of mature trees of conifers has been achieved successfully in many recalcitrant pines throughout the world. Therefore, we conclude that both stress conditions and signaling molecules plays an important role in cloning of mature trees of conifers.

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