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Short Communication

Factors influencing cloning mature trees of conifers

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At present an embryogenic system derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers. This is the major breakthrough in forest biotechnology, and certainly solves the current problems of tree breeding. During cloning of mature conifers, isolated somatic cells under any external stress conditions of cold\heat or chemical are induced to form a somatic embryo. Cloning of mature conifers was influenced by many factors such as pH, carbohydrate source, plant growth regulators, and activated charcoal. The embryogenic cells are very important because they differentiate, and undergo cleavage polyembryony to form somatic embryos at a later time in conifers.

Key words: Activated charcoal, cloning, carbohydrate, forestry, micropropagation.

Somatic embryogenesis is the development of embryo-like structures under *in vitro* conditions on tissues derived from somatic cells (Konar and Nataraja, 1965; Nataraja and Konar, 1970; Malabadi *et al.* 2004; Feher *et al*. 2003; Namasivayam, 2007; Aronen, 2009). Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Namasivayam, 2007). This is in contrast to the zygote in sexual reproduction which is intrinsically embryogenic (Feher *et al*. 2003; Cairney and Pullman, 2007; Malabadi *et al.* 2004; Namasivayam, 2007). In conifers, there are a number of tissues that could be used as the origin of embryogenic tissue but the ability to form this type of tissue or embryonal suspensor masses (ESM) is generally restricted to the most juvenile stages in tree development. In cell cultures, the dividing cells can follow alternative developmental pathways; such as unorganized callus growth, root and shoot initiation or somatic embryo formation depending on both external and internal factors, such as given growth regulator treatments or genetic preconditioning, respectively. Stress conditions, for example temperature-related, hormonal or osmotic, can influence the fate of tissue cultured plants cells i.e. induce either apoptosis or a developmental switch, and

this phenomenon could be utilized for initiation of somatic embryogenesis as well (Feher *et al.* 2003; Namasivayam, 2007). The molecular bases of this metabolic and developmental switch are poorly understood despite extensive research with different tissue culture systems. There is reasonable consensus concerning the mechanism by which cell division is coupled to programming towards embryogenic pathway, but it is currently contentious how a cell is regulated and whether a creation of embryogenic cells and their conversion to somatic embryo is a driving force in plant growth (Feher *et al*. 2003; Namasivayam, 2007).

At present an embryogenic system derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers, and an embryogenic system could be used for genetic transformation studies (Aronen *et al*. 2007, 2008; Aronen, 2009; Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007; Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006; Malabadi et al. 2008a, 2008b; Park *et al.* 2009). Another important advantage of using vegetative shoot apices of mature pines as a starting material for somatic embryogenesis is that cells are actively dividing, hence their higher regeneration capacity, and serve as the best starting material for genetic transformation studies. These cells are generated by the active division of meristematic tissue, and meristimatic cells possess higher regeneration potential. This will also result 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 parents. This review paper highlights the important factors influencing cloning mature trees of conifers, and following are the factors that might play an important role in promoting cloning mature trees of conifers.

1) **Activated charcoal**

The positive role of activated charcoal in medium promotes growth and development of plant tissues (Pan and van Staden, 1998). The beneficiary effects of activated charcoal on tissue responses *in vitro* could be attributed to establishing polarity by darkening the medium, adsorption of inhibitory substances, produced by either the media or explant (Pan and van Staden, 1998). During cloning of mature pines, the use of activated charcoal at 0.3% in the DCR (Pre culture medium) has increased the survival rate of explants of *P. kesiya, P. roxburghii, P. wallichiana, P. patula,* and *P. sylvestris,* and ultimately the resulted in the induction of embryogenic tissue on DCR induction medium (Aronen *et al*. 2007, 2008; Aronen, 2009; Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007; Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006; Malabadi *et al.* 2008a, 2008b). The sole purpose of addition of activated charcoal in the pre- culture medium is to remove all the phenolic exudation and toxic chemicals leached out during *in vitro* cloning of mature pines. The concentration of activated charcoal in the basal medium also plays an important role during cloning of mature pines. Higher concentrations of activated charcoal (0.4, 0.5, 0.6, and 0.7%) have found inhibitory effect on *in vitro* cloning of mature pines. Therefore, the optimum concentration of activated charcoal for the *in vitro* cloning of mature pines is 0.3%, and incubation period of 3 days at 2-4^{o}C.

2) **Carbohydrate source**

Another very important factor that governs the initiation of embryogenic cultures in conifers was the carbohydrate source. During cloning of mature pines, the

tTCL explants completely failed to produce embryogenic callus with sorbitol, mannitol, glucose and fructose. Among the carbon sources tested, sucrose and maltose were found best carbon sources. In case of sucrose, (20 to 40 gl $^{-1}$), two types of calluses were produced viz embryogenic and green non-embryogenic callus. On an average, amount of non-embryogenic callus was more as compared to embryogenic callus. On the other hand, the use of maltose as a carbon source for the initiation and proliferation of embryogenic cultures resulted in higher percentage of white glossy smooth friable mucilagenous embryogenic callus with proembryonal masses during cloning of most of the pines e.g. *P. kesiya, P. roxburghii, P. wallichiana, P. patula,* and *P. sylvestris,* Maltose was therefore, the best carbon source (30 gl $^{-1}$) for the initiation of embryogenic cultures especially for multi step somatic embryogenesis procedures in conifers (Aronen *et al*. 2007, 2008; Aronen, 2009; Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007; Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006; Malabadi *et al.* 2008a, 2008b). In general maltose seems to be superior to sucrose for green plant regeneration from anther and isolated microspore culture systems of grass species. It was thus assumed that beneficial effect of maltose came from its slow hydrolysis.

3) **Plant growth regulators**

Plant growth regulators play an important role in growth and development of plants and signaling too. Auxin regulates cell divisions, differentiation and elongation. The influence of exogenously applied auxins preferentially 2,4-dichlorophenoxy acetic acid 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 (Feher *et al*. 2003; Aronen *et al*. 2007, 2008; Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007; Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006; Malabadi *et al.* 2008a, 2008b). During cloning mature trees of pines, a combination of NAA, 2, 4-D and BA at a particular concentrations induced embryogenic tissue on the DCR induction medium. The optimum concentration the external growth regulators was 22.62 µM 2,4-D, 26.85 µM NAA and 8.87 µM BA, and tTCL (explants) induced embryogenic tissue in many pines e.g. *P. kesiya, P. roxburghii, P. wallichiana, P. patula,* and *P. sylvestris.* Higher or lower concentrations of NAA, 2, 4-D and BA have resulted in the induction of non-embryogenic tissue. Therefore, the dedifferentiation of somatic cell is influenced by many factors like stress conditions, then simultaneous activation of auxin and stress responses may be key event in cellular adaptation, causing genetic, metabolic and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells.

4**) pH:** *physiological competence*

Characteristic changes in intracellular pH are hypothesized to be associated with this transition. One positive marker of cell differentiation and cell activation is the cellular pH (Feher *et al.* 2003; Namasivayam, 2007). A modification and changes in cytoplasmic pH was found to be required for the control of the cell cycle, cell division and growth (Feher *et al.* 2003; Namasivayam, 2007). The pH values in the vacuoles as well as in the chloroplasts may serve as an indicatior of the cell type (embryogenic or non-embryogenic. Increased cytoplasmic pH correlates with cell division, although it is not known wheather cytoplasmic alkalization serves as a mitotic signal or is a consequence of cell activation (Feher *et al*. 2003; Namasivayam, 2007).

Cytoplasmic pH was correlated with cell division. Alkalinization promoted the cell cycle in the meristematic region of the hypocotyls, while acidification inhibited it. Buffering of the pH in a medium abolishes establishment of the cellular pH gradient (Feher *et al.* 2003). The pH of the medium was also found to influence somatic embryo induction and developments in plants. In *Picea abies* cultures, induction rates were higher between pH 6.5 and 7.5 than pH 5 and 6. Medium pH was decreased in these cultures (low to pH 4), which was likely correlated with increased pHc. The development of embryos could only be advanced if the medium pH was raised to approximately pH 5.7. Pro-embryonal masses to somatic embryos transition in cell cultures of Norway spruce is associated with a drop in the pH of the medium and increased programmed cell death (PCD). It was also reported that buffering of medium pH with 10mM MES prevented embryogenic cell formation under inductive conditions.

Conclusion

There are many factors influence the cloning of mature conifers particularly the physiological status of the buds, *in vitro* culture conditions supplemented with external hormones, pH of the medium, activated charcoal and carbohydrate sources. Cloning of mature conifers using *tTCL* of apical meristematic tissue has many practical applications, and could be applied as an efficient micropropagation method for the clonal forestry.

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