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Short Communication

Activation of cambial layer influences cloning of mature trees of conifers

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The shoot apical meristems are organized pools of undifferentiated or embryonic cells (stem cells) maintained by a dynamic balance between cell division and differentiation. On the basis of our mature tree cloning of pines, 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. 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. 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.

Key words: Apical meristem, conifers, cloning, India, somatic embryogenesis, stem cells.

Overview

Cloning of selected superior mature tree is recognized as a powerful tool in forest tree improvement (Malabadi *et al.* 2004; Malabadi *et al.* 2011a, 2011b). 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 (Konar and Nataraja, 1965; Nataraja and Konar, 1970). During this somatic-to embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression system (Feher *et al.* 2003; Malabadi *et al.* 2004; Malabadi *et al.* 2011a, 2011b). 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 (Feher *et al.* 2003; Malabadi *et al.* 2011c). 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

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parents. 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 (Malabadi *et al*. 2004; Malabadi *et al.* 2011a, 2011b).

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 (Feher *et al.* 2003; Malabadi *et al.* 2012). The shoot meristem is the source of all above-ground post-embryonic organs in higher plants. 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 (Malabadi *et al.* 2012). 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 leads to a continuous flow of progeny stem cells (Malabadi *et al.* 2012). 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 by our laboratory and successful in many recalcitrant conifers (Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006, Aronen *et al*. 2007, 2008; Aronen, 2009; Malabadi and Nataraja, 2007; Malabadi *et al*. 2008a, 2008b; Park *et al* 2009; Malabadi and Teixeira da Silva, **2011**).

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. 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. These cells were generally present in small, compact aggregates and showed competence for embryogenesis. These cambial layer cells (stem 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 non-embryogenic tissue in conifers. Microscopic observation of non-embryogenic tissue confirmed loosely arranged, vacuolated and often elongated thin parenchymatous cells with sparse cytoplasm and few starch grains. Such cells did not show morphogenetic competence for somatic embryogenesis in conifers. 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. The non-embryogenic tissue is often mixed with cambial layer cells, and it is very difficult to separate the embryogenic cells.

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 (Ho and Vasil, 1983; Malabadi *et al.* 2010; Malabadi *et al.* 2011d, 2011e, 2011f). In this method, non-embryogenic cells could be largely eliminated by the application of selection procedure and culture of embryogenic cells. 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. On the other hand vacuolated non-embryogenic cells remained in the upper portion. The middle part of the suspension consisting of small clumps of embryogenic cells was used as the inoculums for subculture. 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. 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). The suspension is centrifuged at the lower speed for about 10-15min resulting in the clear separation of two layers. 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. This method is used in our laboratory for the selection of embryogenic cells particularly for the conifer tissue culture experiments. These cells were plated on media under *in vitro* conditions for the induction of somatic embryogenesis in mature conifers. The resulting cultures were composed predominantly of embryogenic cells originated from cambial layer only. 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. At this stage one can not proceed further and all the cultures will be turning brown in spite of successful initiation of somatic embryogenesis. Identification and separation of embryogenic cells is one of the tedious processes during mature tree cloning of conifers. Therefore, activation of cambial layer is very important in the initiation of embryogenic cultures during the cloning of mature conifers. 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. This tissue can not be used for the induction of somatic embryogenesis in conifers since cambial layer cells are not programmed towards embryogenesis. 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. This activation again depends upon many factors such as type of buds collected, timing of buds collection, stress conditions, and also signaling molecules (Malabadi *et al*. 2011b). These factors should be optimized before starting cloning experiments of mature trees of a particular pine species under study. Another important factor is growth cycle of the particular pine species should be studied before starting mature tree cloning experiments. 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. This also largely depends upon the pine species, and type of the stress conditions used, and signal molecules. Hence activation of the cambial layer is species-specific. In our study the tissue produced from activated 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 vitro*

conditions. The activation of cambial region is a very interesting phenomenon during cloning of mature conifers. This again depends upon the transmission of the signal to the cambial region. As per our previous discussion in one of the review paper, 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 (Malabadi *et al.* 2011b). 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. 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. Our group is very much interested to study this signaling phenomenon. The cellular and metabolic pathway of activation signaling should be studied in detail.

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