Research in Plant Biology, 2(2): 43-47, 2012 ISSN : 2231-5101
www.resplantbiol.com

Short communication

Influence of bud break and apical meristematic tissue competence during cloning mature trees of conifers

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This review paper highlights the influence of bud break and apical meristematic tissue competence in inducing embryogenic tissue during cloning mature trees of conifers. Bud burst depends on dormancy release by chilling or heat due to increase in the day temperature, and accumulation of thermal time above a species-specific threshold. 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,* 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 leads to a continuous flow of progeny cells. These cambial layer cells under stress conditions undergo differentiation and leading to the embryogenic pathway in conifers.

Key words: Apical meristem, bud burst, cloning, conifers, mature trees, somatic embryogenesis

Overview

Pine bud break (burst) timing is very important factor, which influences the *in vitro* cloning mature trees of conifers. 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 growth-dormancy cycle, bud break or burst with local climate. The developmental or growth cycle from bud set to bud burst consists of several sub processes-dormany induction, dormancy release and forcing-each with different responses to climatic conditions. Buds undergoing dormancy in winter, cessation of growth in the fall and initiation of growth of buds during early summer are key processes in this life cycle of conifers (Choudhury, 1962; Konar, 1958, 1963; Dormling *et al.* 1968). Therefore, temperature and change in the climate of a particular location influences bud burst timing to a greater extent. In species with a free growth pattern, growth cessation

and cold acclimation are induced by short day and low temperatures in fall. After growth cessation, bud dormancy develops gradually and a state of true dormancy may result after 8-10 weeks. True dormancy can be defined as the inability of a bud to burst at normal growth temperatures in long (Weiser, 1970; Hanninen *et al.* 2007). After dormancy induction, bud burst depends on dormancy release by chilling or heat due to increase in the day temperature, and accumulation of thermal time above a species-specific threshold. In some of the plant species e.g. black alder (*Alnus glutinosa* L) and birch (*Betula* spp), increased temperature in autumn enhances the chilling requirement and delays bud burst, lowering the risk of premature bud burst in later winter and early spring, a risk that may increase in the event of climate warming (Heide, 2003). The timing of cold hardening in plants, onset of dormancy and bud burst are genetically determined that are adapted to local conditions. In case of conifers or trees with a wide range of distribution, the timing of bud set and the development of frost resistance can vary significantly between different provenances or ecotypes of the same species (Choudhury, 1962; Konar, 1958, 1963; Dormling *et al.* 1968; Heide, 2003; Weiser, 1970; Hanninen *et al.* 2007; Repo *et al.* 2000).

In India, there are 4 clear seasonal changes that is rainy season (1st of June to 30th of August), fall (September to November), winter (November to February), summer (March-May) respectively. During winter season (November- February), the shoot buds of *P. kesiya*, *P. roxburghii* and *P. wallichiana* were found dormant and growth of the shoot buds was inhibited by the low temperature in both North and Southern parts of India. The bud burst might be during the early days of summer, showing the first sign of active growth in March or some times in April (depending upon the climate change for the particular year). 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. In *P. roxburghii* the shoot buds collected after the bud break during April have a positive response in terms of inducing embryogenic mass of tissue. In *P. kesiya*, apical shoot buds induced somatic embryo-genesis during the period of April-July. This is due to a change of climate during the particular year during the collection of buds for *in vitro* cloning studies of *P. kesiya*. In *P. wallichiana*, the shoot buds were collected during the month of May has yielded an embryogenic tissue (Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007). Apical meristematic cells may retain a high enough degree of juvenility to make them more amenable to *in vitro* cloning of mature conifers. The active growth of apical shoots of *P. kesiya, P. roxburghii* and *P. wallichiana* immediately after the bud burst is only a very short window (for the collection) for *in vitro* cloning studies. This short period of availability of plant material limits the scale of investigation. The buds collected after bud burst are very active in growth and also successfully induced somatic embryogenesis in *P. kesiya*, *P. roxburghii* and *P. wallichiana* (Malabadi *et al*. 2004; Malabadi and Nataraja, 2006; Malabadi and Nataraja, 2007). On the other hand shoot buds collected during entire year in our *in vitro* cloning of mature pines has resulted in the induction of non-embryogenic tissue might be due to the absence of active internal regulatory signals for somatic embryogenesis. In *P. patula*, the shoot bud material was available during end of March to middle of April and resulted in

the induction of embryogenic tissue mass (Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006). In rest of the collections of the whole year, the apical shoot buds of *P. patula* from South Africa resulted in inducing a non-embryogenic tissue (Malabadi and van Staden, 2003; 2005a, 2005b, 2005c; Malabadi and van Staden, 2006). This is also holds good for *P. sylvestris* from Finland where the bud material is available only during the month of April-May (summer started in Finland). The shoot buds collected before April or after May has yielded the non-embryogenic tissue due to a short window of availability of plant material (Aronen *et al.* 2007, 2008). Therefore, the optimum time for the collection of plant material particularly apical shoot buds for *in vitro* cloning of mature pines is immediately after the bud burst or break, which is always influenced by local climate change in temperature due to the onset of summer season in many countries of the different geographical locations throughout the world. This time frame of availability of bud material is very short, and needs to be identified through the study of growth cycle for a particular pine species before starting the *in vitro* cloning experiments. Although latitudinal patterns of variation have been found in angiosperm trees such as oak (*Quercus* spp.) and birch, the effect of latitude is often confounded with the climatic gradient. It was also estimated that the predicted rise in temperature in this and next century could advance the time of bud burst by 2 to 3 weeks in Norway spruce growing at 64^{o} N, which could increase the risk of injury by spring frosts and indicates the need to identify late-flushing genotypes for planting in areas where spring frosts frequently occur (Hanninen *et al.* 2007).

During our studies on *in vitro* cloning of differnt pines (*P. kesiya, P. roxburghii, P. wallichiana*, *P. patula,* and *P. sylvestris)*, the storage of plant material at 2 to 4°C for 3 days have not affected the induction of embryogenic tissue. On the other hand, storage of bud material more than 3 days has drastically affected the induction of embryogenic tissue, and resulted in the browning of tTCL and discarded. This might be due to a over stress conditions of the bud material during the long-term storage of the bud material. Therefore, for a better and optimum results, the storage of shoot buds either in cold or room temperature should be avoided. Induction of somatic embryogenesis using vegetative shoot apices of mature pines was very successful without storage of bud material in the most of pines e.g. *P. kesiya*, *P. roxburghii*, *P. wallichiana*, *P. patula*. In all our experiments, shoot buds were processed for *in vitro* cloning on the very same collection day, and this has influenced the success rate of *in vitro* cloning to a greater extent than storing the bud material in terms of higher percentage of responsive explants (tTCLs) in inducing embryogenic tissue. Another reason is that during storage, most of the buds were wounded; wounding itself induces the stress and resulted in the browning during initiation of embryogenic tissue, and ultimately resulted in the death of the explants. Therefore, storage of buds has negative effect on the tissue initiation and ultimately resulted in the non embryogenic tissue. Therefore, we conclude that storage of shoot bud material at 2 to 4^{o}C for more than 3 days has resulted in the browning of explants leading to the induction of non embryogenic tissue from mature pines. Hence storage must be avoided and the buds should be processed immediately.

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. Stresses not only promote dedifferentiation, but also can be used to induce somatic embryo formation. 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

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. 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, 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. 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. 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 leads to a continuous flow of progeny cells. 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 non-embryogenic tissue in conifers.

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