Mini Review

Somatic embryogenesis: an alternative method for *in vitro* micropropagation

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Abstract
Plant tissue culture is an alternative method of commercial propagation and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants. Somatic embryogenesis is a process by which asexual or somatic cells are induced to form embryos in culture. Somatic embryogenesis is a multi-step regeneration process starting with formation of pro-embryogenic masses, followed by somatic embryo formation, maturation and regeneration. This paper outlined the important processes involved in the somatic embryogenesis.
Keywords: Somatic embryogenesis; Micropropagation; Somatic embryo; Regeneration; Plant tissue culture.

INTRODUCTION

Plants are unique in their ability to produce somatic embryos (Jayasankar *et al*., 2001). All somatic cells within a plant contain the entire set of information necessary to create a complete and functional plant (Wang, 1997). Somatic embryogenesis is another alternative to traditional vegetative propagation methods as it offers a rapid large-scale propagation system (Fuentes *et al.*, 2000; Find *et al*., 1998; Capuana and Debergh, 1997). Furthermore, the plantlets produced through somatic embryogenesis are reported as true-to-type (Martin, 2003b).

Somatic embryogenesis is a process whereby embryos develop from somatic cells or tissues in tissue culture achieve maturity and subsequently germinate to form normal plants (Witjaksono, 1997). Von Arnold and co-workers (2002) further defined somatic embryogenesis as a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue. Most somatic embryos including those from woody species pass through the same stages of development as a zygotic embryo; the globular, heart-shaped, torpedo- and cotyledonary stage (Sobri *et al.*, 2005; Ramos *et al*., 2003; Choi *et al.*, 2002a; Tejavathi *et al*., 2000; Choi *et al*., 1999). In woody species, the proliferating embryogenic tissues are often secondary embryos (Ibaraki and Kurata, 2001). Secondary somatic embryogenesis is a phenomenon whereby new somatic embryos are initiated from somatic embryos (Vasic *et* al., 2001). Although similar structurally and physiologically, somatic embryos are lack of protective seed coats, nutritive accessory tissues and the quiescent resting phase as in zygotic embryos (Jayasankar *et al*., 2001). Wang (1997) stated that somatic embryos contain embryo specific macro-molecules like storage proteins and lipids.

Somatic embryos can be obtained via either direct or indirect somatic embryogenesis. In direct embryogenesis, the embryos originate directly from tissues in the absence of conspicuous callus proliferation (Aly *et al.,* 2002) whereas indirect embryogenesis involves the callus proliferation and embryogenic tissues precede embryo development (Fuentes *et al*., 2000). Explants from which direct embryogenesis is most likely to occur include ovules, zygotic and somatic embryos, and seedlings (von Arnold *et al*., 2002). Induction of somatic embryos directly from plant tissue is the most desirable approach in order to avoid somaclonal variation (Aoshima, 2005). Furthermore, it reduces the time required for plant regeneration, which may be beneficial to minimize culture-induced genetic changes (Fuentes *et al*., 2000). Efficient systems based

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on direct somatic embryogenesis have been developed for numerous species including alfalfa, forest trees, celery, red clover, *Camellia japonica* and chickpea (Yantcheva *et al*., 1998).

In vitro propagation through somatic embryogenesis has been reported in many medicinal plants such as *Eurycoma longifolia* (Sobri *et al.*, 2005), *Ocimum basilicum* L. (Gopi and Ponmurugan, 2006) as well as in a few spruce and pine species (Haggman *et al*., 2005; Arya *et al*., 2000). For instance, more than 500 somatic embryo plantlets of loblolly pine were successfully established in the field in the year 1991 to 1993 (Tang *et al*., 1998). Somatic embryogenesis of woody plants was achieved in the 1960's with *Citrus* spp., *Biota orientalis*, *Santalum album* and *Zamia integrifolia*; however, it was not until the 1980's that woody plant somatic embryogenesis was more widely reported (Karkonen, 2001). For many applications, somatic embryos have powerful advantages for mass propagation; that is, the high multiplication rate, the ease of use of liquid medium, the handling of enormous numbers of embryos at one time and the possible use of bioreactors (Hamidah *et al*., 1997). Thus, it is a more preferred pathway than organogenesis because it offers better potential for multiplication (Yantcheva *et al*., 1998). Besides, plant regeneration by somatic embryogenesis provides an ideal *in vitro* system for basic studies of plant cell biology and embryo development (Find *et al*., 1998). Somatic embryogenesis also provides an ideal experimental process for investigating differentiation and for understanding the mechanisms of expression of totipotency in plant cells (Arya *et al*., 2000). In addition, the plantlets arising from embryoids have a tap root system which is superior to adventitious roots (Sita, 1986). Thus, somatic embryogenesis can be one of the important tools especially for the plants that do not or have low percentage of tap root formation in the micropropagation system (Sobri *et al*., 2006a).

Stages of somatic embryogenesis: Somatic embryogenesis is a multi-step regeneration process starting with formation of pro-embryogenic masses, followed by somatic embryo formation, maturation, desiccation and plant regeneration (von Arnold *et al*., 2002). Although the somatic embryogenesis protocols largely depend on plant species and cultivars, the production of somatic embryos generally involves four main processes: (1) induction, (2) maintenance of embryogenic cultures, and (3) development of embryos (4) Regeneration of the embryos (Korban, 2005; Ibaraki and Kurata, 2001).

Initiation of embryogenic callus: Embryogenic callus is defined as a type of callus that is either able to produce somatic embryos or is capable of embryogenesis when it is subjected to proper culture conditions (Gairi and Rashid, 2004). It is usually easy to distinguish between embryogenic and non-embryogenic callus based on its morphology and colour (von Arnold *et al*., 2002). According to Sun *et al*. (2003) and Yang *et al*. (2003) embryogenic callus tends to be either (1) compact, yellowish-white with nodular structures and slow growing or (2) highly friable with embryos interspersed among masses of dry loosely organized cells. Meanwhile, non-embryogenic callus also has two distinct types: (1) translucent-to-yellow-to-brown callus with a wet, rough, crystalline, unorganized appearance and composed of rather large elongated cells or (2) highly organized tissue composed of a loose callus matrix with proliferating root primordial (Kebebew *et al.*, 1998; Fei, 1997). In terms of the biochemical content, von Aderkas *et al*. (2002) stated that embryogenic callus contains twice the amount of starch compared to non-embryogenic callus and also overall amino acid content comparable to that of the zygotic embryos. Early identification and selection of somatic embryogenesis in plants can also be carried out using the biochemical markers such as protein and isoenzyme banding profiles (Sobri *et al*., 2006b). Sobri *et al.* (2006b) reported that two proteins at 21 and 25 kDa were highly accumulated in the embryogenic callus of *Eurycoma longifolia*.

Somatic embryos have been induced from a variety of plant tissues, most frequently from zygotic embryos (Corredoira *et al*., 2002), immature inflorescence (Lauzer *et al*., 2000), roots (Chand and Sahrawat, 2002), leaves (Zhang *et al*., 2000), hypocotyls segments (Choi *et al.,* 1999) or directly from zygotic buds or cotyledons (Kevers *et al*., 2002). However, almost all somatic embryos in conifers and deciduous trees are derived from zygotic embryos (Wang, 1997). The conditions under which somatic embryogenesis has been achieved are quite variable for different woody species (Karkonen, 2001). In a majority of studies, auxins, particularly 2,4-D have been employed for the induction of somatic embryos (Umehara and Kamada, 2005; Gumerova *et al*., 2003; Pareek and Kothari., 2003; Mary and Jayabalan, 1997). For example, initiation of embryogenic cultures of some woody species such as *Citrus clementina*, *C. sinensis* and *C. limon* require continuous exposure to 2,4-D (Kayim and Koc, 2006) while in the case of *Gymnema sylvestre*, a combination of 2,4-D and BA managed to produce the secondary embryos (Kumar *et al*., 2002b). In some other cases such as rice (Gairi and

Rashid, 2004) and *Cucumis melo* L. (Kintzios *et al*., 2002), an initial pretreatment of 2,4-D for two to three days is sufficient to induce secondary embryogenesis that will continue for years on a basal medium. Meanwhile, supplementation of other auxin, NAA to the medium enhanced the embryogenic response in another woody species, *Sesbania sesban* var. *bicolor* (Shahana and Gupta, 2002).

Proliferation of embryogenic callus: Embryogenic callus is often accompanied by non-embryogenic callus which grows faster than the embryogenic callus. Thus, repeated selection of embryogenic callus for subculture is critical (Fei, 1997). Subculture interval also has a great impact on maintenance of embryogenic callus. Embryogenic cultures of some species and some genotypes can be subcultured for a prolonged period on medium containing plant growth regulators, and still retain their full embryogenic potential (von Arnold *et al*., 2002). Somaclonal variation has been shown to increase with the prolonged *in vitro* culture (Lakshmanan and Taji, 2000). Normally, embryogenic callus is maintained and proliferated in a medium similar to that used for initiation (Kevers *et al.,* 2002; Anand *et al*., 2000; Selles *et al*., 1999). However, the degree of embryo differentiation which takes place in the presence of auxin varies in different species (Hamidah *et al*., 1997).

Development and maturation of somatic embryos: Somatic embryo formation through callus culture often starts when embryogenic callus is transferred to media whereby the concentration of auxin or auxin-like compounds is reduced or omitted (Umehara and Kamada, 2005; Yang *et al*., 2003) although in some cases, somatic embryogenesis and maturation occur at the same growth regulator concentration (Martin, 2003c). With the depletion of auxin, the block on the expression of those genes required for the transition to the heart shape is removed (von Arnold *et al*., 2002).

One of the most important events occurring during embryo development is the differentiation of the shoot and apical meristems (Stasolla and Yeung, 2003). During the maturation stage, the somatic embryos undergo various morphological (Schuller *et al*., 2000) and biochemical changes (Blanco *et al*., 1997). Incomplete maturation of somatic embryos is one of the most significant factors that account for low rates of plant conversion (Witjaksono, 1997). Somatic embryo maturation can be controlled by treatments with abscisic acid (Blochl *et al*., 2005), sucrose and desiccation (Thomas, 2006; Jheng *et al*., 2006). Other supplements that influence development include osmoticants and gelling medium (von Aderkas *et al*., 2002). Reports of maturation of somatic embryos for various conifers species emphasized the importance of reducing the osmotic water potential of the medium by increasing the concentration of sucrose or supplementing the medium with mannitol, sorbitol or polyethylene glycol (PEG MW 4000) in the presence of abscisic acid (Garin *et al*., 2000).

Germination of embryos and plant conversion: According to Stasolla and Yeung (2003), the terms germination refers only to roots elongation, which may not be necessarily followed by shoot growth while conversion refers to growth of a functional shoot and root system and it is through this process that viable plantlets are produced. Germination of somatic embryos and regeneration into plants has been reported to be low for most species (Jayasankar *et al*., 2001) probably due to the structural abnormality of somatic embryos (Lee *et al*., 2002). In addition, only mature embryos with a normal morphology and which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants (von Arnold *et al*., 2002; Walker and Parrott, 2001).

Somatic embryos usually develop into small plants, comparable to the seedlings, on culture medium lacking plant growth regulators (Stefanello *et al*., 2005; Park *et al*., 2005). However, there are cases where auxin and cytokinin stimulate germination (von Arnold *et al*., 2002). In *Zingiber officinale* Rosc. for instance, supplementation of 3.0 mgl^{-1} BA kinetin in the regeneration medium converted the somatic embryos into plantlets (Guo and Zhang, 2005). Meanwhile, for some species such as *Picea glauca*, inclusion of extra compounds like casein hydrolysate at 1.0 gl^{-1} (w/v) is also required (Garin *et al*., 2000).

Regeneration of cell suspension: Liquid medium processes have been investigated widely with the aim of obtaining synthetic seeds on an industrial scale (Aberlenc-Bertossi *et al*., 1999). However, application of various technologies for plant breeding purposes, viable protoplast and cytoplast fusions, organelle transfer, *in vitro* selection and some molecular techniques rely on the availability of cell suspension cultures which maintain their regeneration potential (Remotti, 1995). Cell suspension cultures are generally initiated from compact or friable callus of which the latter type is generally considered to be the most suitable (Luczkiewicz *et al*., 2002; Figueiredo *et al*.,

2000). The cell suspension culture composed of a heterogeneous population of embryogenic and non-embryogenic cells (Holme, 1998). The embryogenic cells contains a dense cytoplasm showing an evident nucleus and containing starch grains while the non-embryogenic cells were large, vacuolated without an evident nucleus with no cell division (Tribulato *et al*., 1997).

The somatic embryos in the liquid medium underwent similar development stages as in the embryos cultured in the solid medium (Anand *et al*., 2000). The presence of starch, which is often related to the embryogenic cells and is considered to be an indicator of development towards somatic embryos, has also been observed in the embryogenic cell suspension cultures of *Lilium longiflorum* Thunb (Tribulato *et al*., 1997). However, Nhut *et al*. (2006) reported that the number of somatic embryos derived from embryogenic calli of *Lilium longiflorum* cultured in liquid medium, especially at a volume of 20 ml, were shown to be more than in solidified medium, 170 rather than 28, and the forms of somatic embryos in different developmental steps (globular, heart and cotyledon-shaped) were clearly distinguished under microscope. Besides, Choi *et al*. (1999) also observed that the somatic embryos formed on agar medium had larger cotyledons and a more intact epidermis compared to embryos formed in liquid medium. The lower somatic embryo multiplication on solid media may be due to binding of water and absorption of minerals and plant growth regulators by the gelling agents resulting in the depleted supply to the growing embryos (Sood *et al*., 2002).

Unlike the limited reports on somatic embryogenesis in suspension culture of monocots, systems for somatic embryogenesis have been established using suspension cultures in some species of dicotyledons such as carrot, celery and caraway (Kim and Soh, 1996). Several studies involving embryogenic suspension cultures of woody species have also been reported in *Eleutherococcus senticosus* (Choi *et al*. 2002b; Choi *et al*., 1999), *Valeriana edulis* spp. *procera* (Castillo *et al*., 2000), *Picea abies* and *Picea sitchensis* (Find *et al*., 1998). The embryogenic cell line obtained from cell suspension culture will be useful for production of useful secondary metabolite by metabolic engineering using genetic transformation and for cryo-preservation of genetic resources (Kim *et al*., 2003), for large scale multiplication, efficient in vitro selection of putative genetic transformants as well as for the production of non-chimeric transgenics (Thiruvengadam *et al*., 2006).

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