ELSEVIER

Cell Biology International 33 (2009) 524–533

Cell Biology International

www.elsevier.com/locate/cellbi

Establishing *in vitro Zinnia elegans* cell suspension culture with high tracheary element differentiation

Peter Twumasi [a,b,c,*], Jan H.N. Schel [a], Wim van Ieperen [b], Ernst Woltering [d], Olaf Van Kooten [b], Anne Mie C. Emons [a]

[a] *Laboratory of Plant Cell Biology, Department of Plant Sciences, Wageningen University and Research centre, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands*

[b] *Horticultural Production Chains Group, Department of Plant Sciences, Wageningen University and Research Centre, Marijkeweg 22, 6709 PG Wageningen, The Netherlands*

[c] *Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana*

[d] *Agrotechnology and Food Innovation, Wageningen University and Research Centre, Wageningen, The Netherlands*

Received 3 January 2009; accepted 31 January 2009

Abstract

The *Zinnia elegans* mesophyll cell culture is a useful system for xylogenesis studies. The system is associated with highly synchronous tracheary element (TE) differentiation, making it more suitable for molecular studies requiring larger amounts of molecular isolates, such as mRNA and proteins and for studying cellulose synthesis. There is, however, the problem of non-uniformity and significant variations in the yields of TEs (%TE). One possible cause for this variability in the %TE could be the lack of a standardized experimental protocol in various research laboratories for establishing the *Zinnia* culture. Mesophyll cells isolated from the first true leaves of *Z. elegans* var Envy seedlings of approximately 14 days old were cultured *in vitro* and differentiated into TEs. The xylogenic culture medium was supplied with 1 mg/l each of benzylaminopurine (BA) and α-naphthalene acetic acid (NAA). Application of this improved culture method resulted in stable and reproducible amounts of TE as high as 76% in the *Zinnia* culture. The increase was mainly due to conditioning of the mesophyll cell culture and adjustments of the phytohormonal balance in the cultures. Also, certain biochemical and cytological methods have been shown to reliably monitor progress of TE differentiation. We conclude that, with the adoption of current improvement in the xylogenic *Z. elegans* culture, higher amounts of tracheary elements can be produced. This successful outcome raises the potential of the *Zinnia* system as a suitable model for cellulose and xylogenesis research.

Keywords: Cellulose; *In vitro* culture; Programmed cell death; Apoptosis; Tracheary element; Xylogenesis; *Zinnia elegans*

1. Introduction

Xylem vessels and tracheids are important structures in higher plants due to their water conducting abilities and mechanical support (Tyree, 2003). The xylem cells originate from the root and shoot procambium during the early developmental stages of the plant and also from the vascular cambium during the secondary growth periods of the plant. For over a century, extensive work has been done to unravel the complex mechanisms involved in xylem formation and its hydraulic function in the plant (Aloni, 1987; Chaffey, 1999;

Abbreviations: BA, benzylaminopurine; CM, culture medium; DAPI, 4,6-diamidino-2-phenylindole; DIC, differential interference contrast; FDA, fluorescein-diacetate; ICM, inductive medium; NAA, α-naphthalene acetic acid; NICM, non-inductive medium; PCD, programmed cell death; TE, tracheary element; TUNEL, Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling; VE, vessel element.

* Corresponding author at: Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology (KNUST), PMB, Kumasi, Ghana. Tel.: +233 245 131806; fax: +233 516 4338.

E-mail address: twumasipeter@gmail.com (P. Twumasi).

1065-6995/$ - see front matter © 2009 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2009.01.019

Dengler, 2001). In just over two decades, our knowledge about xylogenesis both at cellular and molecular levels has increased more than ever before (McCann et al., 2001). For instance, there is a great deal of studies involving xylem formation focusing on understanding the mechanism of cellulose synthesis (Haigler et al., 2001; Mellerowicz et al., 2001; Cano-Delgado et al., 2003). The availability of xylogenic cell culture systems, such as *Zinnia*, *Arabidopsis* and *Populus*, has provided essential tools for an in-depth understanding of the xylogenesis process (Ye, 2002). Programmed cell death (PCD) is essential during formation of certain functional structures, in both animals and plants. It is also involved in defense mechanisms as demonstrated in the hypersensitive response (Iakimova et al., 2008). In animals this type of self-induced cell death is referred to as apoptosis, a term relating to the apoptotic bodies (or membrane-bound structures) resulting from the breakdown of the membrane at the end of the death process (Yang et al., 1999; Ranganath and Nagashree, 2001; Sanmartin et al., 2005). In animals, these apoptotic bodies are later engulfed through phagocytotic activity in the organism. Such apoptotic bodies have, however, not been found in plant cells during programmed cell death. Because of the involvement of the PCD in TE differentiation in the xylem (Fukuda, 1996; Roberts and McCann, 2000), more attention is being focused on the use of this simpler xylogenic system to study regulation of PCD in plants.

The *in vitro* xylogenic cell culture is suitable for the study of xylem development and differentiation studies due to the easy accessibility for manipulation, microscopic analyses and production of one simple cell type isolated from the complexity of the tissue. On the other hand, the whole plant xylogenic process is preserved. Since many molecular studies require extraction of ample amounts of the molecule under study, a highly efficient and synchronous TE differentiating cell culture would be necessary. The *Zinnia elegans* xylogenic cell culture, although introduced long ago (Fukuda and Komamine, 1980), continues to show the highest efficiency and synchrony in TE differentiation better than any of the recently introduced xylogenic cultures (*Arabidopsis*: (Oda et al., 2005); Populus: (Ohlsson et al., 2006)). At the time of *Zinnia* xylogenic cell culture discovery, the yield of TE was just around 30% (Fukuda and Komamine, 1980). More recently, there have been records of TE differentiation as high as 60% (Church, 1993; Fukuda, 1996).

Despite these achievements, different laboratories report of low and varying TE yields formation in the xylogenic *Zinnia* cultures (Gabaldon et al., 2005; Tokunaga et al., 2005; Oda and Hasezawa, 2006). The inconsistencies in the protocol for establishing the *Zinnia* culture, ranging from plant material to phytohormonal induction, may account for these differences. The aim of this work therefore was to produce a standardized and reproducible protocol for establishing higher yields of TEs in *Z. elegans in vitro* cultures. Also, modified cytological and biochemical methods were to be designed for monitoring progress of TE differentiation.

2. Results

2.1. Sequence of events during TE differentiation

Mechanically isolated *Zinnia* mesophyll cells have definite shapes, usually asymmetrically cylindrical and measuring 20–60 μm in length. We have established in this work that phytohormone application 24–48 h after cell isolation produces TE differentiation (24-h induction, 74% TE; 48-h induction, 76% TE). Also, a reliable viability measurement was achieved within this period. Viability of cells in the NICM observed over 5 days showed no significant changes, but the change was significant in the ICM (Fig. 2). Cells in the ICM maintained higher cell division as compared to those in NICM (Fig. 3). However, cells from ICM were smaller as compared with those from NICM (Fig. 4). Expansion of cells in the ICM occurred within the first 24 h of the culture while in the NICM the expansion continued beyond 48 h. In the ICM, this stage was immediately followed by an active secondary cellulose deposition on the cell wall leading to formation of cellulose bands.

TE differentiation becomes visible 48 h after treatment. This stage is characterized by sequential secondary cellulose cell wall deposition, nuclear condensation, vacuole rapture and DNA laddering. Finally, autolysis of the differentiating cells revealed various secondary cellulose band patterns. Various types of tracheary elements present *in planta* were recovered in the *in vitro* culture (Fig. 5). Lignification occurred later around 120 h in the inductive cultures, about 12 h from complete autolysis of the TEs. The sequence of events that occurred during TE differentiation in the *Zinnia* culture is schematically summarized in Fig. 6.

2.2. Vital staining with FDA

We observed that high levels of dead mesophyll cells in starting *Zinnia* culture either inhibited TE differentiation completely or significantly reduced TE differentiation. Moreover, since mature TEs are dead and hollow cells with secondary cellulose thickenings, the TE differentiation itself eventually results in lowering of the initial viability. Cell viability is therefore a measure of the progress of TE differentiation. Differentiating *Zinnia* cultures with initial cell viability of 60% or higher (Fig. 7) was found to produce workable amounts of TEs.

2.3. Nuclear condensation, DNA labelling with TUNEL and gel electrophoresis

At 48 h of the culture, the nuclei of cells in ICM were condensed –losing the regular oval or round shape which is visible with DAPI staining. The nuclei of the differentiating cells disappeared between 72 and 96 h period of the culture, a stage associated with extensive autolysis of the differentiating cells. These phenomenal changes were however not present in control cultures (Fig. 8I, J).