Progress on coconut micropropagation in Mexico

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Introduction

The coconut palm has always been a very important species for man. In recent years its commercial importance has been growing very rapidly for different high value products, as in the case of packed coconut water, since it has the potential to substitute worldwide bottled carbonated drinks with a healthier offer. Giant corporations in this field, Coca-Cola, PepsiCo and Dr. Pepper, are already selling packed coconut water products in USA and Europe. According to www.canadeanconsumer.com there will be a fourfold increase (from 2.9 to 10 billion USD) in the coconut water value growth by year 2019.

This increasing growth of the coconut industry markets needs a corresponding growth in nut production worldwide. This is a task difficult to achieve considering the threat of several pests and diseases, and most importantly because most coconut plants in producing countries are old. Regarding phytosanitary threats, perhaps the most worrying are the devastating phytoplasma diseases. In the Americas the phytoplasma associated Lethal Yellowing disease (LY) has killed millions of palms in different countries in the Caribbean region (Fig.1). Therefore, important efforts have been carried out in Jamaica and México to identify LY resistant coconut, which have successfully identified resistant ecotypes in both countries (Oropeza *et al.*, 2005).

Thus in order to maintain the flourishing markets and growing demand of coconut products, replanting of most cultivation surface around the world, as well as establishing new surface, are urgently needed. It is estimated that this immense task cannot be accomplished by traditional propagation through seed. Accordingly, the biotechnological alternative of in vitro propagation by somatic embryogenesis, with its great propagation capacity, has been approached in different laboratories worldwide in order to develop highly efficient and commercially viable protocols. This paper reports an account of such an effort that is currently going on in México at CICY (Centro de Investigación Científica de Yucatán). For more in depth background information the reader is referred to excellent reviews that are available (Arunachalam, 2012; Nguyen *et al.*, 2015; Sáenz-Carbonell *et al.*, 2013).

Figure 1. Lethal Yellowing, the phytoplasma associated disease, has killed millions of coconut palms in the Americas.

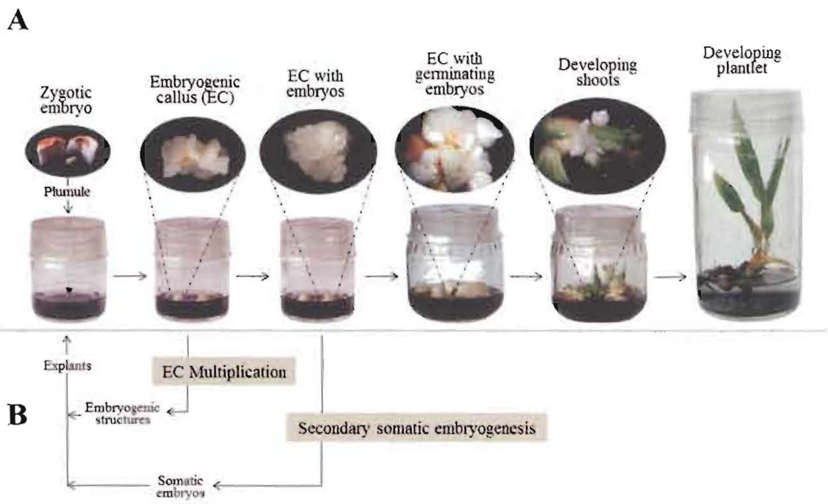

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Figure 2. Diagram of the process of plant regeneration from plumule explants via somatic embryogenesis (A). Modified process including embryogenic callus multiplication and secondary somatic embryogenesis (A and B).

Materials and Methods

All the materials and methods used for the research reported here are described in Chan *et al.*, (1998), Pérez-Núñez *et al.*, (2006), Sáenz *et al.*, (2006), Pérez-Núñez *et al.*, (2009) Sandoval-Cancino *et al.*, (2016).

Results and Discussions

Development of a protocol using plumule explants

Early studies were carried out during the nineties within a collaboration of Wye College (UK) and CICY (Blake et al, 1994), testing different explants that initially included rachilla and whole embryos for formation of callus (that could eventually form somatic embryos with the capacity to convert into viable plantlets). The callus formation response of these explants was not very convincing, however whole embryos showed the formation of a ring of callus-like tissue growing on the outside middle part of the embryo, although it did not develop into a proper callus. Then considering this response and the probable occurrence of inhibitors of callus formation in some embryo tissues (Kefeli et al., 1971), parts of the embryo were tested separately as explants, including the plumule. Preliminary results showed that plumules were very responsive for callus formation (Blake *et al,* 1994; Hornung, 1995).

Characterization of embryogenic callus development

Within the next decade basic research was carried out to study the process of somatic embryogenesis from plumule explants with different approaches (morpho-histological, physiological, biochemical and molecular) in order to gain knowledge that could be useful to further improvement of the process. In this way, it was learnt about uptake of 2,4-D by explants and the timeline of how its concentration increased (Sáenz *et al.*, 2005), followed by increases of kinase activity associated with signal transduction (Islas-Flores *et al.*, 2000) and relevant gene expression (Pérez-Núñez et al., 2009; Sáenz *et al.*, 2013). At the same time studies were carried out to characterize morphologically and histologically the development of the embryogenic callus (Sáenz *et al.*, 2006). It was learnt that the development of this callus is precisely well defined, with the formation of ear-shaped translucid structures that start appearing at about 30 days of culture and are fully formed by day 60. These structures have meristematic cells in the periphery tissues. From these tissues, embryogenic structures develop, first with a globular shape and then they become elongated, and that by day 90 of culture the embryogenic callus is fully developed. The embryogenic structures also have meristematic cells in the peripheral tissues, from which somatic embryos develop after transfer to medium designed to induce this response. It is interesting that when the translucid structures start forming there had been already a peak in 2,4-D concentration, on kinase activity and in the expression of CnSERK, an ortholog of the SERK gene (Pérez-Núñez *et al.*, 2009). Also another peak of CnSERK expression happens by day 90 when the embryogenic structures are formed. A very useful finding was that it is possible to follow the proper development of an embryogenic callus just by looking at its morphology following the right pattern of development in form and time, knowing that the correct changes in tissues, and physiological, biochemical and molecular events are taking place as learnt from the basic studies described above. Once embryogenic calli is subcultured to a medium for inducing somatic embryo formation, globular embryos appeared by day 15 and it developed into torpedo-shaped embryos by day 30. Interestingly, there is a peak of CnSERK activity by day 15.

Figure 3. Scaling up of the process of coconut micropropagation in a facility outside Mérida, Yucatán, with a capacity for up to 200,000 plantlet production per year.

In parallel studies, more practical approaches were tested including changes in the media formulation to study the effect of activated charcoal and brassinosteroids (Azpeitia *et al.*, 2003) on plumule explants, and gibberellins (Montero-Cortés *et al.*, 2010) and BAP (Montero-Cortés *et al.*, 2011) on embryogenic structures used as explants, on the formation of embryogenic callus, somatic embryos and germination, resulting in improved efficiency.

Multiplication of embryogenic callus

The characterization of embryogenic callus, described above, lead us to believe that embryogenic structures and globular somatic embryos could be useful as explants because of the presence of meristematic cells and expression of CnSERK. Therefore they were tried as explants and results were positive for both. These results allowed us to develop processes of callus multiplication / secondary somatic embryogenesis (Pérez-Núñez *et al.*, 2006) (Fig. 2B). The first intended for massive multiplication and the second (as an intercalated step within the multiplication) to help conserve embryogenic competence during prolonged culture times (Martinelli et al. 2001). This combined approach was tested with results showing an estimated capacity to produce about one hundred thousand somatic embryos from a single plumule explant (Pérez-Núñez *et al.*, 2006). Embryos were able to germinate and convert to plantlets that after planting grew successfully to sexual maturity and fruit production. This protocol is currently being scaled up to a semi-commercial level in a facility we call "Bio-fábrica" or biofactory in Sierra Papacal nearby Mérida in Yucatán (Fig. 3).

Somatic embryogenesis from rachilla explants

Also within the past five years using rachilla explants, a protocol was developed for the production of embryogenic callus and its multiplication, embryos were able to germinate and convert to plantlets (Sandoval-Cancino *et al.*, 2016). These results are setting the basis to develop a process for massive propagation of coconut; similar to the one already developed using plumule explants, and using the knowledge gained from the plumule studies.

Conclusion and Perspectives

The transference of the technology for massive propagation based of multiplication of embryogenic callus from plumule is underway and working well, with embryogenic callus and embryo yields as expected, the full process will be working with production of plantlets during the second semester of 2017. There are plans at a later stage, to establish in México larger facilities, probably five. In parallel we are working on the establishment of embryogenic callus lines of other coconut ecotypes of interest. Keeping these will require cryopreservation, so it is planned to start working on this issue as soon as possible. Also we are already working for developing embryogenic callus lines derived from rachilla explants of very valuable genotypes, and this will be followed by testing them for massive production with a process similar to that used for plumule derived calli. However, it is clear that although results have been very satisfactory so far, for future strengthening of the coconut micropropagation efficiency capacity, it is necessary to keep on working for continuous improvement of protocols. This is something that certainly requires a multi-institutional, international, and very well organized and coordinated effort. ■ ** Reproduced from Cocoinfo International 24 (1), 2017*

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