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Coconut Micropropagation in Mexico using Plumule and Floral Explants

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Abstract

This paper focuses on the research efforts carried out by CICY in Mexico on micropropagation of coconut. They started during the nineties in collaboration with Wye College (UK) and ORSTOM-CIRAD (France), with the development of a protocol that was reproducible and more efficient than previous ones, based on plumule explants grown in different media based on Y3 medium added with activated charcoal, gelling agent and of particular importance growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). Within the next decade basic research was carried out to study the process of somatic embryogenesis from plumule explants, with an approach including morpho-histological, physiological, biochemical and molecular points of view, in order to gain knowledge that could be useful to further improvement of the process. Also different practical approaches were tested including changes in the media formulation, embryogenic callus multiplication and secondary somatic embryogenesis. As a result a highly efficient protocol was developed that could potentially yield over a hundred thousand somatic embryos from a single plumule explant. Embryos were able to germinate and convert to plantlets, that after planting, successfully grew to sexual maturity and fruit production. This protocol is currently being scaled up to a semi-commercial level. Also within the past five years, a protocol using rachilla explants has been developed for the production of embryogenic callus and its multiplication, and embryos produced were able to germinate and convert to plantlets, setting the basis to develop a process for massive propagation of coconut, such as the one already developed using plumule explants.

Keywords: Coconut; Micropropagation; Somatic Embryogenesis; Plumule; Rachilla

Introduction

The coconut palm has always been a very important species for man. In recent years this importance has been growing commercially a lot. Such as the case of packed coconut water, among other high value products, since it has the potential to substitute 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 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 production. 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

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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 Mexico 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 market and growing demand of coconut products, replanting of most cultivation surface worldwide, as well as establishing new surface, are urgently needed. It is believed that this immense task cannot be accomplished by traditional propagation through seed. Therefore the biotechnological alternative of *in vitro* propagation by somatic embryogenesis, with its great propagation capacity, has been approach in different laboratories worldwide to try to develop highly efficient and commercially viable protocols. This paper reports an account of such an effort that is currently going on in Mexico at CICY. 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).

Materials and methods

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

Results and discussion

Development of a protocol using plumule explants

Early studies carried out during the nineties CICY (Mexico) in collaboration with Wye College (UK) and ORSTOM-CIRAD (France), different explants were tested including whole embryos. The use of plume derived from: (a) observations with whole embryos used as explants, in which there was a ring of callus-like tissue growing on the outside middle part of the embryo but that did not develop into a proper callus; and (b) the probable occurrence of inhibitors of callus formation in some of the embryo tissues (Kefeli*et al*., 1971).

According to these it was believed that a potential for callus formation was present in the embryo, but probably not as whole embryo but as isolated parts, so different parts were tested and preliminary results showed that plumules were found to be very responsive (Hornung, 1995). After further research, a protocol developed by testing different media based on Y3 medium (Eeuwens 1976) added with activated charcoal, gelling agent and different combinations of growth regulators 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) at different concentrations, andalso subculture intervals were tested (Chan *et al*., 1998) (Fig. 2A). Production of embryogenic callus was obtained from plumule, somatic embryos formed on the calli germinated and converted into plantlets. The resulting protocol was reproducible and more efficient than previous ones and results could be quantified (Chan *et al*., 1998).

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 an approach that included morpho-histological, physiological, biochemical and molecular points of view, in order to gain knowledge that could be useful to further improvement of the process. 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 the development of the embryogenic callus morphologically and histologically (Sáenz *et al*., 2006). It was learnt that the development of this callus is very 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. From

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

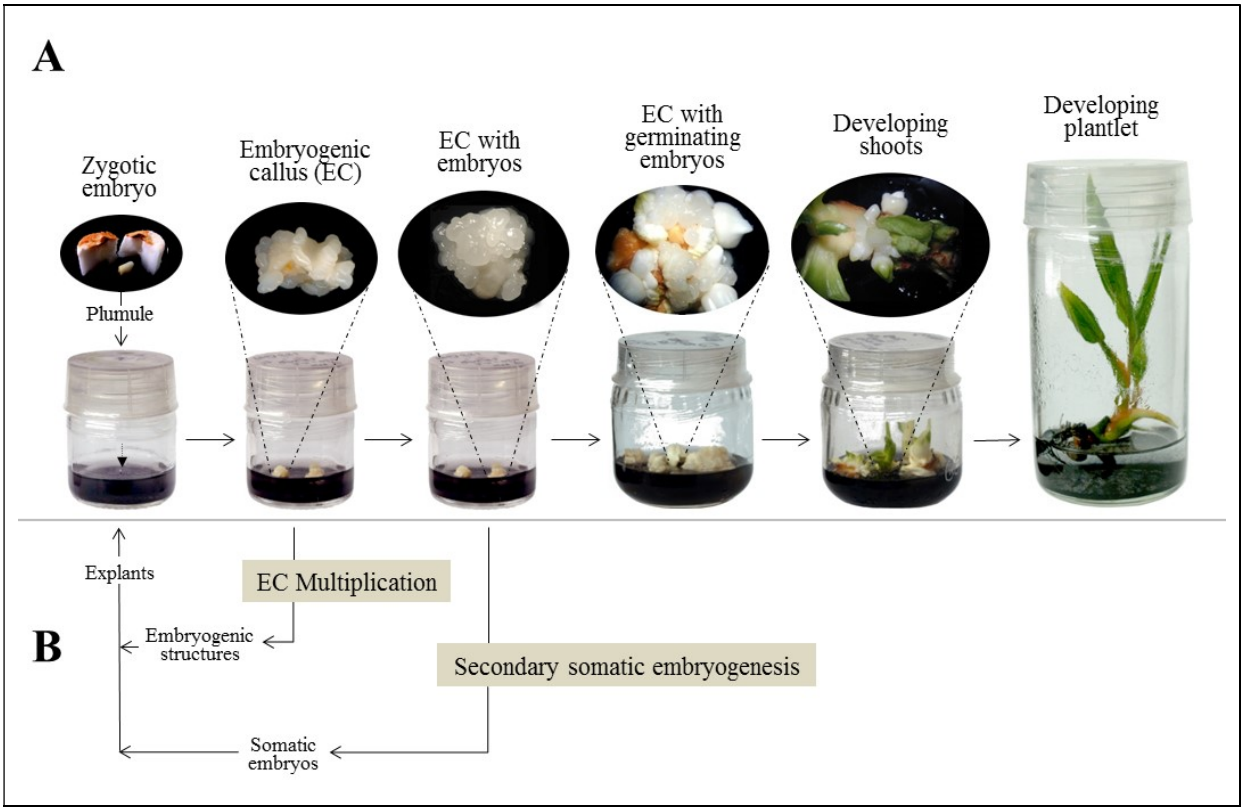

Figure 2. Diagram of the process of plant regeneration from plumule explants via somatic embryogenesis (**A**). Modified process including embryogenic callus multiplication and secondary somatic embryogenesis (**A** and **B**).

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

these, embryogenic structures develop first globular and then 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 therehad been already a peak in 2,4-D concentration, on kinase activity and in the expression of *CnSERK*, anortholog 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 in form and time, knowing that all the correct changes in tissues, and physiological, biochemical and molecular events are taking place as learnt from the basic studies. Once embryogenic calli is subcultured to a medium for inducing somatic embryo formation, globular embryos appeared by day 15 and the developed into torpedo-shaped embryos by day 30. Interestingly there is a peak of *CnSERK* activity by day 15.

In parallel studies, more practical approaches were tested including changes in the media formulation to study the effect activated charcoal andbrassinosteroids (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/or expression of *CnSERK*. They were tried and it worked for both. These results allowed us to develop processes of callus multiplication and 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 a 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 levelin 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 this experience.

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 callus and embryo yields as expected, we will have the full process working with production of plantlets during the second semester of 2017. Then it is planned to establish larger facilities, probably five in Mexico. In parallel we are working on the establishment of embryogenic callus lines of different ecotypes of interest. Keeping these will this require cryopreservation, so it is planned to start this as soon as possible. Also we are already working of developing embryogenic callus lines derived from rachilla explants of very valuable plants, and this will be followed by testing massive production with the same process that is being used for plumule derived calli. Although results have been very satisfactory it is necessary to keep on working for continuous improvement of coconut micropropagation. This most certainly will require a multi-institutional and international effort.

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