25

Somatic Embryogenesis in Recalcitrant Plants

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1. Introduction

There are two types of embryogenesis in plants: zygotic and somatic (Figure 1). Zygotic embryogenesis is one of the most important steps in the life cycle of plants. The process begins with double fertilization, followed by determination of the three axes of embryos (longitudinal, lateral, and radial) and morphologic changes of the embryos (globular, heart-shaped, and torpedo-shaped; Figure 1). Subsequently, seed storage proteins accumulate in the embryos, and finally, the embryos become desiccated and dormant. These processes are regulated by numerous factors, including phytohormones, enzymes, and other substances related to embryogenesis.

Somatic embryogenesis (SE) is the process by which somatic cells, under induction conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes (Quiróz-Figueroa *et al*., 2006), that result in the production of bipolar structure without vascular connection with the original tissue. The development of somatic embryos closely resembles the development of zygotic embryos both morphologically and physiologically (Figure 1). The process is feasible because plants possess cellular totipotency where by individual somatic cells can regenerate into a whole plant. Since the first reports on carrot in 1958 (Reinert 1958; Steward *et al*., 1958), somatic embryogenesis has been reported in various plant species.

In addition to natural *in vivo* forms embryogenesis (apomixis), there exist at least three ways to induce embryo development from *in vitro* cultured plant cells: *in vitro* fertilization, from

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microspores and *in vitro* somatic embryogenesis (Féher *et al*., 2003). *In vitro* SE can develop either from callus (indirect SE) or directly from the explant without any intermediate callus stage (direct SE). Somatic embryogenesis is also induced directly, or through callus, in the culture of somatic embryos, and this process is called secondary SE in contrast to primary SE induced from explant cells (Gaj, 2004).

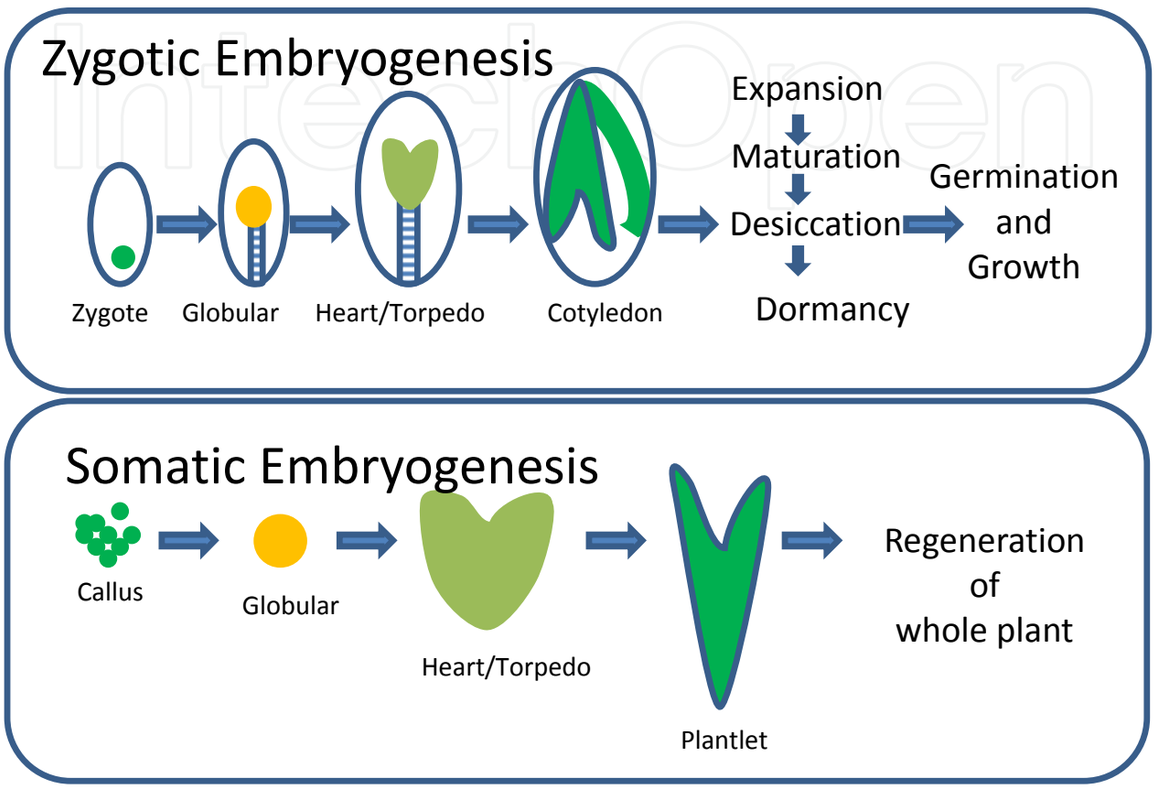

Fig. 1. Zygotic vs somatic embryogenesis. Modified from Zimmerman, 1993.

Somatic embryos originate by two pathways, unicellular or multicellular. When embryos have a unicellular origin, coordinated cell divisions are observed and the embryo sometimes connected to the maternal tissue by a suspensor-like structure. In contrast, multicellular-origin embryos are initially observed as a protuberance, with no coordinated cell divisions observable, and those embryos in contact with the basal area are typically fused to the maternal tissue (Quiroz-Figueroa *et al*., 2006).

Somatic embryogenesis with a low frequency of chimeras, a high number of regenerates and a limited level of somaclonal variation (Ahloowalia, 1991; Henry *et al*., 1998) is more attractive than organogenesis as a plant regeneration system (e.g., in genetic transformation, *in vitro* mutagenesis and selection). However there are several factors that influence the initiation of somatic embryogenesis in plants.

In the context of this paper, *in vitro* plant recalcitrance is defined as the inability of plant tissue cultures to respond to *in vitro* manipulations. In its broadest terms, tissue culture recalcitrance also concerns the time-related decline and/or loss of morphogenetic competence and totipotent capacity (Benson, 2000).

2. Some factors influencing somatic embryogenesis induction

The process of acquisition of embryogenic competence by somatic cells must involve reprogramming of gene expression patterns as well as changes in the morphology, physiology, and metabolism of plant cells. Studies on factors controlling *in vitro* plant morphogenesis are highly important not only for the development of improved regeneration systems, but also for the analysis of molecular mechanisms underlying plant embryogenesis.

In vitro development of cells and tissues depends on different factors such as: genotype, type of plant, age and developmental stage of an explant, physiological state of an explant-donor plant, and the external environment which includes composition of media and physical culture conditions (light, temperature) (Gaj, 2004).

The embryogenic potential is largely defined by the developmental program of the plant as well as by environmental cues (Féher, 2005). The key role of endogenous hormone metabolism affected by genetic, physiological and environmental cues is well accepted in the induction phase of somatic embryogenesis (Jiménez, 2005).

The cells which represent an intermediate state between somatic and embryogenic cells are called competent. Cellular competence is associated with the dedifferentiation of somatic cells that allows them to respond to new developmental signals. It is well accepted that embryogenic competent cells can be morphologically recognized as small, rounded cells with rich cytoplasm and small vacuoles. In this respect they are very similar to meristematic cells or zygotes and this similarity is further emphasized by their asymmetric division (Féher, 2005).

Wounding, high salt concentration, heavy metal ions or osmotic stress positively influenced somatic embryo induction in diverse plant species (reviewed by Dudits *et al*., 1995). 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 (Dudits *et al*., 1995).

Endogenous hormone levels however can be considered as major factors in determining the specificity of cellular responses to these rather general stress stimuli (Féher *et al*., 2003). The temporal and spatial changes in endogenous auxin levels are important factors controlling the embryogenic cell fate (Féher *et al*., 2003).

Among different external stimuli that induce an embryogenic pathway of development plant growth regulators (PGRs) such as auxins and cytokinins used for *in vitro* media have been the most frequently considered, as they regulate the cell cycle and trigger cell divisions (Francis and Sorrell, 2001). The high efficiency of 2,4-dichlorophenoxy acetic acid (2,4-D) for induction of embryogenic response found in different *in vitro* systems and plant species indicates a specific and unique character of this PGR. This synthetic growth regulator and an auxinic herbicide appear to act not only as an exogenous auxin analogue but also as an effective stressor (Gaj, 2004).

The polar transport of auxin in early globular embryos is essential for the establishment of bilateral symmetry during plant embryogenesis. Interference with this transport causes a failure in the transition from axial to bilateral symmetry and results in the formation of embryos with fused cotyledons (Liu *et al*., 1993).

The chromatin remodelling plays two major roles during the early stages of somatic embryogenesis. Differentiation requires unfolding of the supercoiled chromatin structure, in order to allow the expression of genes inactivated by heterochromatinization during differentiation, and subsequent chromatin remodelling can result in the specific activation of a set of genes required for embryogenic development (Féher *et al*., 2003).

Also a wide and complex variety of molecules can now be enlisted, including polysaccharides, amino acids, growth regulators, vitamins, low molecular weight compounds, polypeptides, etc. (Chung *et al*., 1992). Some such compounds are derived from the cell wall, whereas others originate inside the cells (Quiroz-Figueroa *et al*., 2006).

3. Some genes related to somatic embryogenesis

3.1 *WUSCHEL* (WUS)

WUS is a homeobox gene which encodes a transcription factor that regulates the pool of stem cells in the shoot meristem and is regulated by a feedback loop involving the CLAVATA (CLV) genes (Weigel and Jurgens, 2002; Bhalla and Singh, 2006). WUS expression can be first localized to the shoot meristem in the heart stage embryo, and the shoot meristem of the plant by regulating the stem cell pool can continue to produce organs throughout the life of the plant. The stem cells are specified by a WUS-dependent signal produced in the organizing center cells, which lie below the stem cell niche of the central zone and CLV3 is in turn produced by the stem cells of the central zone (Baurle and Laux, 2005; Reddy and Meyerowitz, 2005). Increases in the number of stem cells lead to an increasing amount of the secreted CLV3 protein, which acts via the CLV1/CLV2 receptor complex to reduce WUS expression and the number of stem cells, thus maintaining a constant pool of stem cells (Weigel and Jurgens, 2002).

3.2 *Baby Boom* (BBM)

The Baby Boom (BBM) gene, which was isolated from microspore embryo cultures of *Brassica napus* (Boutilier *et al*., 2002), encodes a transcriptional factor belonging to the AP2/ERF family. BBM expression was observed during zygotic and pollen-derived somatic embryogenesis. The ectopic expression of BBM and *Arabidopsis* BBM (AtBBM) in transgenic plants induced the formation of somatic embryo-like structures on the edges of cotyledons and leaves, as well as additional pleiotropic phenotypes, including neoplastic growth, phytohormone-free plant regeneration from explants, and abnormal leaf and flower morphology. Therefore, BBM is likely to promote cell proliferation and morphogenesis during embryogenesis (Boutilier *et al*., 2002).

3.3 *SERK* (Somatic Embryogenesis Receptor Kinase)

Among the genes involved in somatic embryogenesis, Somatic Embryogenesis Receptor Kinases (SERKs) genes has been detected in the early stages of the process, which form a subgroup in the Leucine-Rich Repeat-Receptor-Like Kinases (LRR-RLKs) comprising the largest subfamily of RLKs in plants and are also related to key processes in plant growth (Sharma *et al*., 2008).

The first SERK gene was identified in competent cells of carrot (*Daucus carota*) *in vitro* cultured (Schmidt *et al*., 1997), this gene encodes a transmembrane receptor kinase type with leucine-rich repeat (LRR). DcSERK has been considered as a marker of cells competent to form embryos in culture (Schmidt *et al*., 1997). DcSERK has been found be expressed in somatic and zygotic embryos but in no other plant tissues at very early stages of somatic embryo development, i.e., from the single-cell stage to the globular stage (Schmidt *et al*., 1997). Genes homologous to DcSERK were isolated from *Arabidopsis* (AtSERK1), maize (ZmSERK1, ZmSERK2), *Medicago truncatula* (MtSERK1) (Nolan *et al*., 2003), *Hieracium* (HpSERK), *Helianthus annuus* (Thomas *et al*., 2004), *Oryza sativa* (Hu *et al*., 2005), *Theobroma cacao* (Santos *et al*., 2005), Citrus unshui (Shimada *et al*., 2005), y *Solanum tuberosum* (Sharma *et al*., 2008) suggesting the ubiquity of a small family of SERK in all species of plants, in addition to the functional conservation of a specific role in embryogenesis. Their expressions were detected during somatic embryogenesis (Somleva *et al*., 2000; Baudino *et al*., 2001; Hecht *et al*., 2001; Shah *et al*., 2002; Nolan *et al*., 2003; Tucker *et al*., 2003; Thomas *et al*., 2004), as well as in developing ovules and early-stage embryos of *Arabidopsis*, *Hieracium* and maize.

4. Genetic transformation to abate recalcitrance

Genetic transformation has proven to be an alternative to abate recalcitrance to *in vitro* morphogenesis and to increase resistance to pathogenic microorganisms (Cai *et al*., 2003; Shin *et al*., 2002; Zuo *et al*., 2002; Herrera-Estrella *et al*., 2004). This has been achieved by insertion and over-expression of genes related to the control of morphogenesis, such as the heterologous gene WUSCHEL in *Arabidopsis thaliana* and *Coffea canephora* cultures that promoted the transition from vegetative to embryogenic state, and eventually led to somatic embryo formation (Zuo *et al*., 2002; Arroyo-Herrera *et al*., 2008). In *Capsicum chinense*, the induced expression of WUSCHEL in segments of transformed stems began to form globular structures, suggesting that heterologous WUSCHEL was active and involved in the process of morphogenesis (Solís-Ramos *et al*., 2009). It has been demonstrated in *Arabidopsis*, that over-expression of a SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK) gene (AtSERK1) increases the embryogenic competence of callus derived from transformed seedlings 3 to 4-fold when compared with the wild-type callus (Hecht *et al*., 2001).

Most of the important crops and grasses are recalcitrant for *in vitro* culturing, which hampers the development of reliable regeneration techniques. This document is focused in the somatic embryogenesis of recalcitrant plants, showing the particular cases of two plant species: habanero chili (*Capsicum chinense* Jacq.) and coconut palm (*Cocos nucifera* L.).

5. Studies in habanero chili (*Capsicum chinense* Jacq.)

5.1 Introduction

All chili peppers belong to the genus *Capsicum* of the Solanaceae family and are important horticultural crops. Members of the *Capsicum* genus have been shown to be recalcitrant to differentiation and plant regeneration under *in vitro* conditions, which in turn makes it very difficult or inefficient to apply recombinant DNA technologies via genetic transformation aimed at genetic improvement against pests and diseases (Ochoa-Alejo and Ramírez-Malagón, 2001). *Capsicum chinense* Jacq. (habanero chili) (Fig. 2-G), a species of economic

importance for Mexico is no exception (Santana-Buzzy *et al*., 2005; López-Puc *et a*l., 2006), and no efficient, reproducible somatic embryogenesis regeneration system has yet been developed for this species. A dependable system is indispensable for their genetic improvement and regeneration of transformed tissue (Solís-Ramos *et al*., 2009).

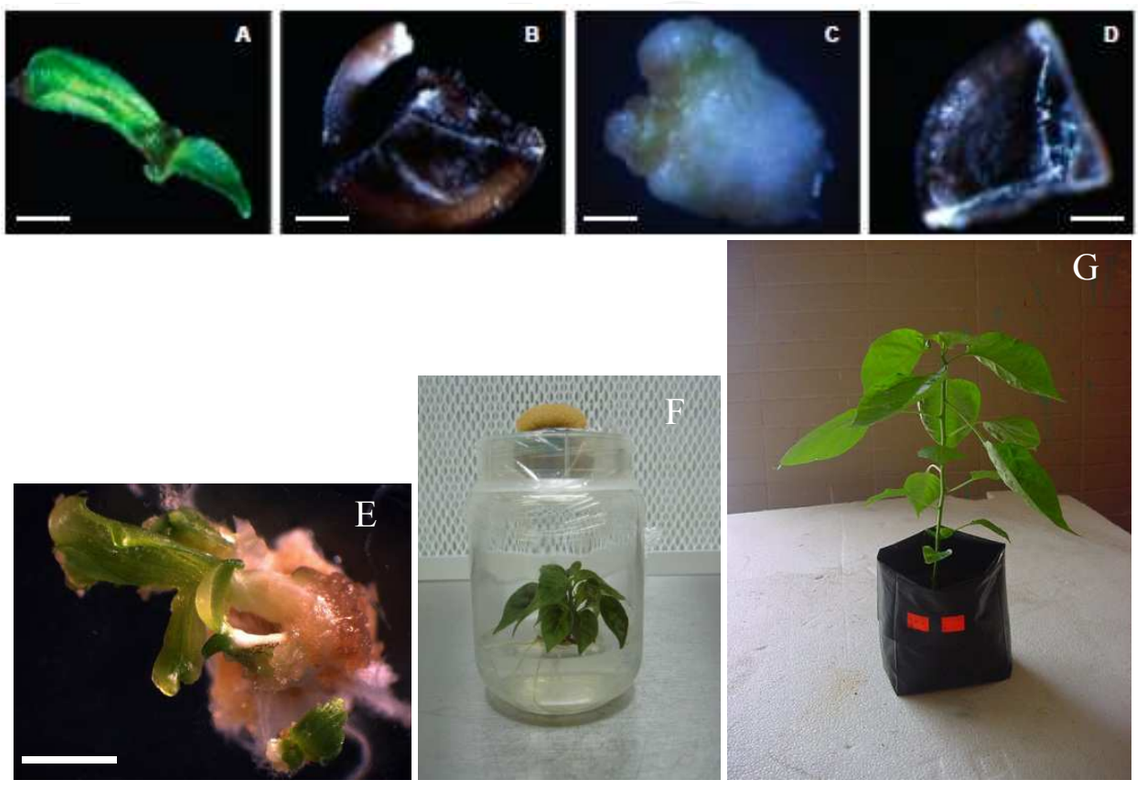

Fig. 2. A to G: Different responses of *C. chinense* explants after induction: A, cotyledon. Bar= 1mm; B, Zygotic embryo segment with radicle; C, Zygotic embryo segment forming callus; D, Zygotic embryo segment without embryogenic response. Bar= 0.5mm. E: somatic embryo germinating. Bar= 1mm. F: seedling obtained from somatic embryo. G: seedling after acclimatization under greenhouse conditions.

5.2 Indirect somatic embryogenesis protocol

Direct organogenesis has been the most frequently used morphogenic route for *in vitro* regeneration of *Capsicum* plants; however, the major problem faced to achieve this goal has been the failure of elongation of the induced shoot buds (Ochoa-Alejo and Ramírez-Malagón, 2001). Shoot buds and rosettes are not well formed during the induction step, perhaps because of a lack of true apical meristems (Binzel *et al*., 1996; Ochoa-Alejo and Ramírez-Malagón 2001; Steinitz *et al*., 2003).

In recent years, a number of investigators have developed methods in order to increase the efficiency of the somatic embryogenesis process for chili pepper micropropagation via direct somatic embryogenesis (DSE) (Harini and Sita 1993; Binzel *et al*., 1996; Khan *et al*., 2006) and

indirect somatic embryogenesis (ISE) (Binzel *et al*., 1996; Buyakalaca and Mavituna, 1996, Kintzios *et al*., 2001, Zapata-Castillo *et al*., 2007, Solís-Ramos *et al*., 2010b). *Capsicum chinense* Jacq. is a recalcitrant species for *in vitro* morphogenesis, and up to date there is no efficient system for genetic transformation and regeneration of this species via somatic embryogenesis. However an ISE protocol was developed using mature *C. chinense* zygotic embryo segments (ZES) (Solís-Ramos *et al*., 2010b) (Figure 2 C, E-G). The ZES cultured in semi-solid MS-3R medium (MS medium with 8.9 μM NAA, 11.4 μM IAA and 8.9 μM BAP) developed an embryogenic callus and 8% of these explants developed somatic embryos (Figure 2-E). Torpedo-stage somatic embryos were detached from the callus and subcultured in semi-solid MS medium without growth regulators, producing a 75% conversion rate to plantlets with well-formed root tissue. Histological analysis showed the developed structures to have no vascular connection to the source tissue and to be bipolar, confirming that this protocol induced formation of viable somatic embryos from mature *C. chinense* zygotic embryo segments, and seedlings can be obtained (Figure 2 F-G).

5.3 Endogenous GUS-like activity in *C. chinense* tissues

The gene *uidA* codes for β-glucuronidase which is utilized as a reporter in plant genetic transformation because it is generally believed that higher plants do not show GUS-like endogenous activity (Jefferson *et al*., 1987; Martin *et al*., 1991; Sudan *et al*., 2006). However, several studies have demonstrated that some plant species show endogenous GUS-like activity in vegetative tissues as well as reproductive organs (Cervera, 2005; Sudan *et al*., 2006). Therefore, in order to avoid undesirable effects in interpreting genetic transformation results, it is recommended to evaluate potential endogenous GUS-like activity in tissues that will be targeted to genetic transformation by using *uidA* as a reporter. The pH of the assay buffer is very critical for detection of the GUS activity in plants. The *E. coli*-derived GUS has optimum activity at pH 7.0 and hence plant tissues are assayed at neutral pH after transformation (Sudan *et al*., 2006).

Segments of mature zygotic embryos of *C. chinense* were used as explants for transient transformation with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA2301) and C58C1 (pER10W-35S Red) (Solís-Ramos *et al*., 2010a, Solís-Ramos *et al*., 2010b). T-DNA in pCAMBIA2301 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) includes a copy of *Escherichia coli uidA* gene under the control of CaMV35S promoter and the NOS terminator. In this binary vector, *uidA* gene coding sequence is interrupted by a Castor Bean catalase intron, which has to be removed for eukaryotic expression and prevents bacterial transcriptions of the gene coding sequence. Transient transformation of *C. chinense* explants and plant regeneration were carried out following the protocol previously described by Solís-Ramos *et al.* (2009). In addition, as a positive control leaves explants of *Nicotiana tabacum* were transient transformed via *A. tumefaciens* LBA4404 (pCAMBIA2301), to verify that the protocol used for GUS activity was done properly. Histochemical staining of *C. chinense* explants was carried out following a protocol reported by Jefferson (1987). Presence of blue spots was recorded and interpreted as transient GUS expression (Figure 3). Also the transient expression of red fluorescent protein was detected using a Leica MZFLIII stereoscopic microscope equipped with appropriate filtres (546/10 nm, 600/40 nm).

Successful transient transformed *C. chinense* zygotic embryo explants were achieved with *A. tumefaciens* LBA4404 (pCAMBIA2301) and the bacteria were eliminated with 1 g/L cefotaxime and 500 mg/L timentin (Solís-Ramos *et al*., 2009). The calli of *C. chinense* transient transformed with pER10W-35S Red (used as control for transformation efficiency) expressed the red fluorescent protein (DsRFP), but not the non-transformed calli (data not shown) (Solís-Ramos *et al*., 2010a). A screening for endogenous GUS-like activity in *C. chinense* tissues was performed in phosphate buffer adjusted to pH 6, 7, 7.5 and 8. At pH 6 and 7 the 100% of all samples (vegetative and reproductive tissues) presented endogenous GUS-like activity (Figure 3-C) (Solís-Ramos *et al*., 2010a). At pH 7.5 no GUS-like activity was observed in all of the petals, root, stem or leaves. However, in septum, stamen and calli some GUS-like activity was observed. A substantial decrease, or even a total absence, of GUS-like activity was observed in phosphate buffer pH 8 in almost all tissue analyzed with an exception for a slight activity in stamens (Figure 3-A) (Solís-Ramos *et al*., 2010a). Our results of histochemical staining in phosphate buffer pH 8, suggest that *uidA* gene was introduced in regenerants of *C. chinense* and *N. tabacum* and the gene was transcriptional active as it can be inferred from the blue stain observed in tissues of regenerated plantlets. The main problem during initial steps of transformation is just to get an assay conditions which can provide an initial screening. This problem has been solved by adjusting the pH to 8 for *C. chinense* (Solís-Ramos *et al*., 2010a).

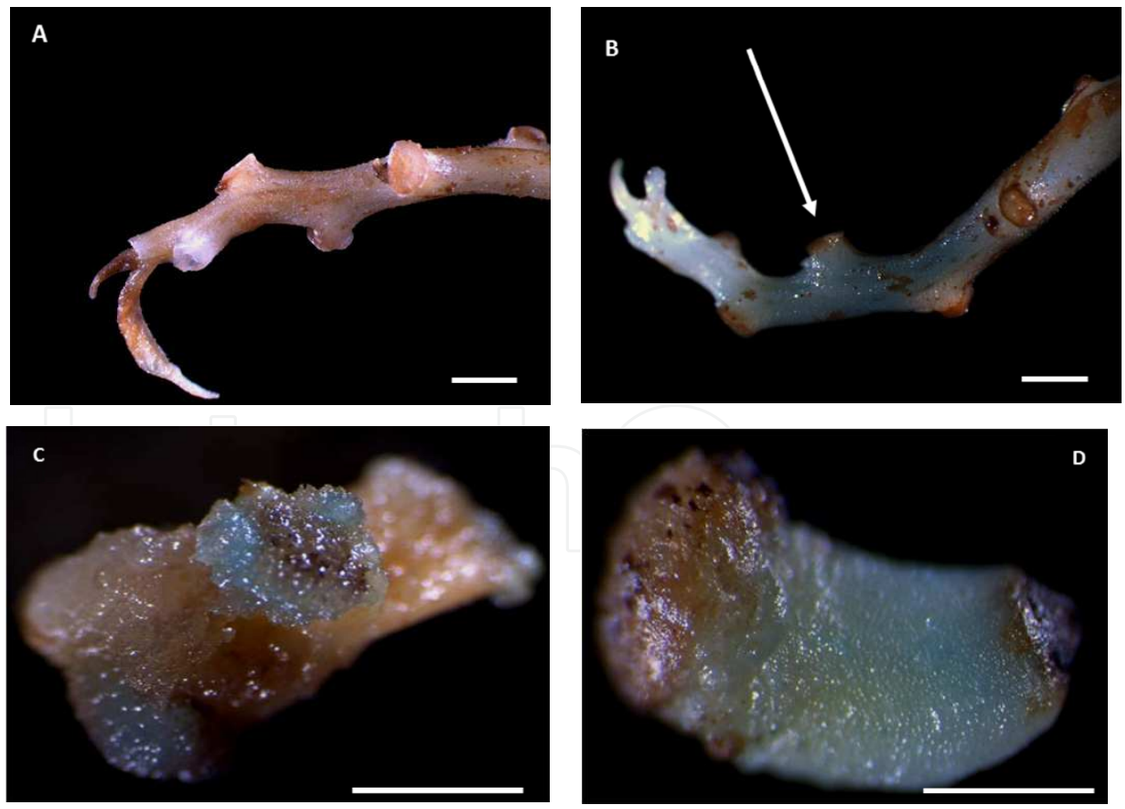

Fig. 3. *C. chinense* explants showing endogenous GUS-like activity at pH 7 (C), and without endogenous GUS-like activity at pH 8 (A). B and D transformed explants showing GUS expression. A, B bar= 5mm. C, D bar= 1mm.

5.4 Protocol for genetic transformation

Habanero chili plants were transformed via *Agrobacterium tumefaciens* co-cultivation with reporter genes: *uiDA*, *DsRFP*, and *WUSCHEL* (Solís-Ramos *et al*., 2009, Solís-Ramos *et al*., 2010a, Solís-Ramos *et al*., 2010b). *WUSCHEL* (WUS) has been shown to promote the transition from vegetative to embryogenic state when overexpressed in *Arabidopsis thaliana* (Zuo *et al*., 2002). The hypothesis tested is that the genetic transformation of Habanero chili and overexpression of heterologous gene *WUS* will promotes an embryogenic response in this species (Solís-Ramos *et al*., 2009). The transformed chimeric plants where used for induction of expression of heterologous gene *WUS*. After 15 days of induction, the segments of transformed stems begun to form globular structures, and the wild type did not show development, suggesting that heterologus *WUS* was active and involved in the process of morphogenesis. The induced transformed explants showed the expression of *WUS* by Northern reverse analysis, and none *WUS* transcripts had detectable in the wild type. The histological analysis of induced transformed stems showed the development of meristematic nodules and the formation of globular somatic embryos, which presented necrosis after 45 days of in vitro culture, which did not continue development into other embryonic stages or in plants. The results showed that overexpression of gene *WUS* in stems of Habanero chili promote the formation of embryogenic structures but these stagnate in their growth suggesting that other signals may be need it for induction of proper development in this species (Solís-Ramos *et al*., 2009). In addition this suggests that *WUS* encourages the development of undifferentiated tissue in species that may help as an alternative to solve the recalcitrance from this plant species (Solís-Ramos *et al*., 2009).

6. Studies for coconut (*Cocos nucifera* L.) somatic embryogenesis

6.1 Introduction

Coconut (*Cocos nucifera* L.) is widely distributed throughout the humid tropics where it is cultivated over an estimated twelve million ha. It is a very important perennial crop, since it significantly contributes to food security, improved nutrition, employment and income generation. Coconut is a monospecific palm species consisting of numerous ecotypes and hybrids all possessing desirable agronomic properties. There is a great ethnic diversity in the ways that various coconut resources are produced and used (Foale, 2005). It is often referred to as "the tree of life" because of the many uses that have been developed for all parts of the palm. More recent uses of economic importance include fibre-derived products for the automobile industry; activated charcoal; virgin oil; bottled water; and oil for production of coco-biodiesel. In the Philippines, an industrial plant was launched in 2006 for the production of 75 million liters / year of coco-biodiesel where it is being used as a fuel additive (Lao, 2009). A blend at 2% coconut oil with diesel has been shown to reduce harmful exhaust emissions (opacity, K value) by as much as 63% (Lao, 2008).

However, most coconut groves require replanting because of loss due either to palm senescence or to diseases such as lethal yellowing in America (Harrison and Oropeza, 2008), the lethal diseases in Africa (Eden-Green, 1997) and cadang-cadang in Asia (Hanold and Randles, 1991). Unfortunately, improved disease resistant planting materials are scarce and seed propagation does not yield sufficient material to satisfy the rapidly growing demands.

Therefore, alternative approaches for the propagation of improved planting materials must be considered and *in vitro* propagation or micropropagation *via* somatic embryogenesis seems to provide a convenient alternative for the future due to its potential for massive propagation.

Several explants have been tested with diverse results, being the most responsive immature infloresencenes and plumules in increasing order (Blake and Hornung, 1995; Chan *et al*., 1998; Pérez-Nuñez *et al*., 2006). For this reason plumules have been more extensively used to improve on the different developmental changes in the process: callogenesis, embryo formation, germination and conversion.

6.2 *In vitro* culture of coconut palm

6.2.1 Coconut micropropagation using plumule explants

In order to increase the efficiency of somatic embryogenesis in coconut, two different approaches were evaluated, secondary somatic embryogenesis and multiplication of embryogenic callus. Primary somatic embryos obtained from plumule explants were used as explants and formed both embryogenic callus and secondary somatic embryos. The embryogenic calluses obtained after three multiplication cycles were capable of producing somatic embryos. The efficiency of the system was evaluated in a stepwise process beginning with an initial step for inducing primary somatic embryogenesis followed by three steps for inducing secondary somatic embryogenesis followed by three steps for embryogenic callus multiplication, and finally production of somatic embryos from callus (Pérez-Nuñez *et al*., 2006). The actual process of somatic embryogenesis by embryogenic callus multiplication and secondary somatic embryogenesis is shown in Figure 4. The total calculated yield from one plumule was 98,000 somatic embryos (SEs). Comparing this to the yield obtained from primary somatic embryogenesis results in about a 50,000-fold increase (Pérez-Nuñez *et al*., 2006).

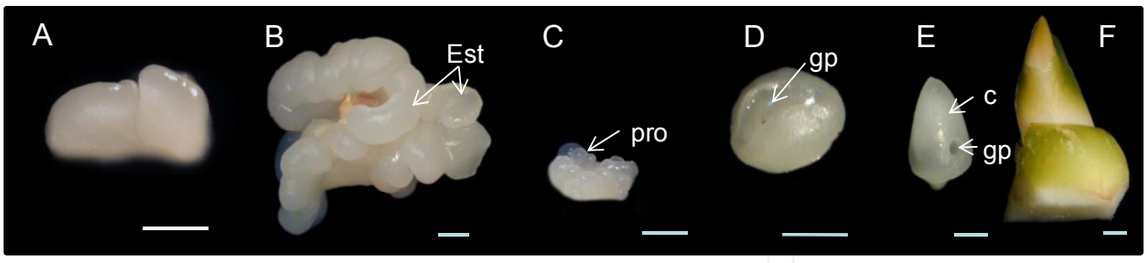

Fig. 4. An embryogenic structure derived from an embryogenic callus used as explant (A), developed an embryogenic callus (B) after 90 days of culture in medium I. After transferring embryogenic callus to medium II, callus with somatic embryos at different stages occurred. Piece of callus with pro-embryos (C), globular embryo (D), coleoptilar embryo (E) and germinating embryo (F). Bar= 1mm. Coleoptile: [c], germinative pore: [gp], pro-embryo [pro]

This protocol represented an important progress towards practical application by showing a way to improve the efficiency of coconut somatic embryo production. However has still some bottlenecks, as the relative low percentage of formation of embryogenic calli (40-

60%) and calli with somatic embryos (12-24%) and the low number of somatic embryos formed (2-10) per callus. In order to increase these figures and optimize this protocol to avoid many steps of multiplication, different plant growth regulators and compounds has been tested.

6.3 Exogenous plant growth regulators

6.3.1 Brassinosteroids

The effect of the brassinosteroid 22(S), 23(S)-homobrassinolide on initial callus, embryogenic callus and somatic embryo formation in coconut plumule explants was tested. The explants were exposed (during a 3 or 7 d pre-culture) to different concentrations (0.01, 0.1, 1, 2 and 4 μM) of the brassinosteroid. The explants responded favorably to the brassinosteroid increasing their capacity to form initial callus, embryogenic callus and somatic embryos. The largest amount of somatic embryos formed, 10.8 somatic embryos / explant, was obtained exposing the explants for 3 d to the brassinosteroid at 0.01 or 0.1 μM, whereas 3.8 somatic embryos / explant were obtained from untreated explants. Efficiency-wise the overall effect of HBr increases the total amount of somatic embryos formed per explant 2.8 times (Azpeitia *et al*. 2003).

6.3.2 Gibberellic acid

In some reports of coconut the GA_3 is added into the culture medium to promote the germination of somatic embryos (Perera *et al*., 2009). However the effect of addition of this phytohormome had not been tested on the formation of somatic embryos. The results obtained with GA_3 were positive at 0.5 μM using the protocol of embryogenic calli multiplication from plumule explants. This concentration promoted 1.5 fold the number of the embryogenic calli forming somatic embryos. The number of somatic embryos per callus also increased, about 5 fold at day 30 (globular embryos) and 2 fold afterwards (coleptilar embryos). Also when the effect of GA_3 was evaluated on the germination of somatic embryos, the results were positive. The proportion of calli with germinating embryos was 2 fold higher than in the control treatment with no phytohormone. The number of germinating somatic embryos *per* callus was also higher under phytohormone treatment, also a 2 fold increase in relation to the control treatment. Therefore, a combined 4 fold increase in the overall number of germinating embryos (Montero-Cortés *et al*., 2010). Then altogether, the use of GA_3 was positive both for the formation of somatic embryos and on their germination, so this could be a very useful approach to improve the performance of coconut micropropagation.

6.3.3 Uptake of auxins

6.3.3.1 Uptake of 2, 4-D

As previously reported for inflorescence coconut explants (Oropeza and Taylor, 1994), ^{14}C-2,4-D was taken up by plumular explants. The rate was faster during the first week of culture, and then reduced until reaching a plateau at day 90. The ^{14}C-2,4-D concentration in the explants reached its maximum values within the first 20 d of culture, prior to the appearance of any morphogenic response. It is interesting to note that when radioactivity was steadily taken up, calli were formed and once the calli started to form embryogenic

structures, uptake practically stopped. This result suggests that the uptake of 2,4-D may be related to the induction of these morphogenic responses.

6.4 Characterization of genes related to somatic embryogenesis

6.4.1 Shoot apical meristem formation and maintaining (*KNOX* family genes)

The expression the class I *KNOX* (KNOTTED-like homeobox) genes seem to play an important role during somatic embryogenesis. In *Picea abies* overexpression of *HBK3,* a class I *KNOX* homeobox gene improves the development of somatic embryos and lines in which *HBK3* was down-regulated had reduced ability to produce immature somatic embryos and were not able to complete the maturation processes (Belmonte *et al*., 2007).

The complete sequences of two *KNOX* like genes were obtained *CnKNOX1* and *CnKNOX2.* The deduced aminoacid sequence of both showed the highly conserved domains characteristic of *KNOX* genes. *CnKNOX1* showed high homology with KNOX class I proteins. *CnKNOX1* expression was detected throughout the embryogenesis process except in somatic embryos at the pro-globular stage, becoming highest in somatic embryos at the coleoptilar stage. No detection of *CnKNOX1* expression occurred in calli with aberrant embryos. The addition of gibberellic acid stimulated the expression of *CnKNOX1* earlier and the relative expression at all stages was higher. *CnKNOX*2 expression occurred at all stages peaking at globular stage but gibberellic acid treatment decreased expression (Montero-Cortés *et al*., 2010).

6.4.2 Somatic embryogenesis (Somatic Embryogenesis Related Kinase-*SERK*)

Somatic embryogenesis involves different molecular events including differential gene expression and various signal transduction pathways for activating or repressing numerous genes sets (Chugh and Khurana, 2002). Genes involved in somatic embryogenesis are stage specific and one of the genes identified in early somatic embryogenesis is SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (DcSERK) that was originally isolated from embryogenic cells in suspension cultures of the dicot *Daucus carota* (Schmidt *et al*., 1997). It was found to be expressed in embryogenic but not in non-embryogenic cultures, in cells predicted to be embryogenic, in tissue explants induced by placing them under embryogenic culture conditions, and during somatic embryogenesis up the globular stage. During *D. carota* zygotic embryogenesis, SERK expression occurred up to the early globular stage, but no expression was found in any other plant tissues, and cells transformed with a SERK promoter-luciferase reporter gene were able to form somatic embryos (Schmidt *et al*. 1997). Similar findings have been obtained in other dicots. In *A. thaliana*, the *AtSERK1* gene was expressed during the formation of embryogenic cells in culture, early embryogenesis, and in plant in developing ovules, specifically in all cells of the embryo sac up to fertilization, and in all cells after fertilization of the developing embryo until the heart stage (Hecht *et al*., 2001). *A. thaliana* seedlings overexpressing AtSERK1 exhibited a three- to fourfold increase in efficiency for initiation of somatic embryogenesis; therefore, an increase in the level of the AtSERK1 conferred embryogenic competence in culture (Hecht *et al*., 2001).

The complete sequence one *SERK* like gene was obtained and referred as *CnSERK*. Predicted sequence analysis showed that CnSERK encodes a SERK protein with the domains reported

in the SERK proteins in other species. These domains consist of a signal peptide, a leucine zipper domain, five LRR, the Serine- Proline-Proline domain, which is a distinctive domain of the SERK proteins, a single transmembrane domain, the kinase domain with 11 subdomains and the C terminal region. Analysis of its expression showed that it could be detected in embryogenic tissues before embryo development could be observed. In contrast it was not detected or at lower levels in non-embryogenic tissues, thus suggesting that *CnSERK* expression is associated with induction of somatic embryogenesis and that it could be a potential marker of cells competent to form somatic embryos in coconut tissues cultured *in vitro* (Pérez-Nuñez *et al*., 2009).

6.5 Protocol for genetic transformation

We have developed a protocol for genetic transformation of this palm species (Andrade-Torres *et al*., 2011); evaluating reporter genes, transformation methods, and conditions for the use of antibiotics to select transformed plant cells. The gene *uidA* was first used for *A. tumefaciens* mediated transformation of coconut embryogenic calli. However, endogenous GUS-like activity was found in calli not co-cultured with bacteria. Then essays for *Agrobacterium*-mediated transformation were developed using green and red fluorescent genes. Both genes are suitables as reporter genes for coconut transformation. In order to establish a protocol for coconut genetic transformation, an approach was used that combined biobalistics to generate micro-wounds in explants, vacuum infiltration and co-culture with *A. tumefaciens* (C58C1+ pER10W-35SRed containing the embryogenesis related gene *WUSCHEL*). Calli treated with the combined protocol showed red fluorescence with greater intensity and greater area than calli treated with either biobalistics or infiltration, followed by bacteria co-culture. PCR amplification of DNA extracts from transformed embryogenic callus produced a band with the expected size using *WUSCHEL* primers (862 bp). No band was obtained using the *Vir*E2 primers. This is the first report of transient genetic transformation of *C. nucifera* and it is the first step toward a protocol that will be useful for the study of the role of genes of interest and for practical applications, such as the improvement of coconut micropropagation via somatic embryogenesis (Andrade-Torres *et al*., 2011).

7. Conclusions and perspectives

The majority of the mechanisms that regulate plant embryogenesis still remain to be clarified. In the higher plants, some genes and factors related to important mechanisms of embryogenesis are plant-specific. The availability of model systems of plant somatic embryogenesis has created effective tools for examining the details of plant embryogenesis. However, studies that used no model plants for somatic embryogenesis systems also revealed the molecular mechanisms in charge of controlling the expression of some genes during somatic embryogenesis, and with practical applications. So the molecular mechanisms of plant embryogenesis might be clarified by experiments using somatic and zygotic embryogenesis either from model or not model plants.

Numerous protocols on successful somatic embryogenesis induction and plant regeneration in different plant species, published last years, suggest that nowadays SE can be achieved for any plant provided that the appropriate explant and culture treatment are employed

(Gaj, 2004). A prerequisite for the successful establishment of a SE system is a proper choice of plant material -the explants being a source of competent cells, and, on the other hand, determination of physical and chemical factors which switch on their embryogenic pathway of development (Gaj, 2004).

The process of acquisition of embryogenic competence by somatic cells must involve reprogramming of gene expression patterns as well as changes in the morphology, physiology, and metabolism (Namasivayam, 2007). These alterations reflect dedifferentiation, activation of cell division and a change in cell fate.

Although few genes have been associated with embryogenesis induction, the search for genes involved in embryogenesis, such as SERK (Hecht *et al*., 2001), LEC (Lotan *et al*., 1998; Stone *et al*., 2001), BABY BOOM (Boutilier *et al*., 2002), WUSCHEL (Zuo *et al*., 2002), and PICKLE (Ogas *et al*., 1999), is a major field of research today (Quiroz-Figueroa *et al*., 2006).

The characterization and functional analysis of protein markers for somatic embryogenesis offer the possibility of determining the embryogenic potential of plant cells in culture long before any morphological changes have taken place, and of gaining further information on the molecular basis of induction and differentiation of plant cells (Tchorbadjieva *et al*., 2005).

The genetic transformation is certainly an important goal to facilitate genetic improvement against several diseases caused by phytopathogenic fungi, bacteria, and viruses, as well as for improvement against different pests (Ochoa-Alejo and Ramírez-Malagon, 2001). However, development of a reproducible tissue culture regeneration protocol is the first step in utilizing the power and potential of this new technology. The system established for *Capsicum chinense* is a promising alternative for cell or transformed plant regeneration through indirect somatic embryogenesis, and may contribute to genetic improvement of *C. chinense* Jacq. by incorporating reporter and interest genes (Solís-Ramos *et al*. 2009; Solís-Ramos *et al*, 2010a; Solís-Ramos *et al*., 2010b).

The protocol for micropropagation of coconut from plumule explants based on embryogenic callus multiplication provides an option not available before for massive propagation of coconuts (Figure 5). However, although it allows the propagation of the progenie of known selected palms, it cannot be used for the cloning of palm individuals with known desirable agronomic traits. On the other hand, the recent developments to obtain embryogenic callus and somatic embryos from immature ovary and anther explants (Perera *et al*., 2007; 2008; 2009), provide an opportunity to try to use these calli as a source of explants (the embryogenic structures) an integrate them into the callus multiplication scheme used with plumule explants. This has already been attempted in CICY using also floral tissue explants, but in this case rachillae slices from immature inflorescences (Oropeza and Chan, unpublished results). The callus obtained was tested for multiplication and although it responded poorly at the beginning though a series of multiplications the percentage of callus formation from embryogenic structure explants was above 40%. Therefore, although preliminary, this is a very promising result that shows that massive propagation from somatic tissue explants from adult plants is attainable in the near future. Finally we should continue with the studies to understand somatic embryogenesis in coconut. The study of genetic control is central for this purpose; therefore it is very important to learn more about the role of those genes that have been isolated and to extend the study to other genes and

components of the genetic control of somatic embryogenesis. The study of these processes, will allow us not only to understand a phenomenon but it might open new avenues of opportunity for further improvement for a more efficient and better quality clonal propagation of coconuts.

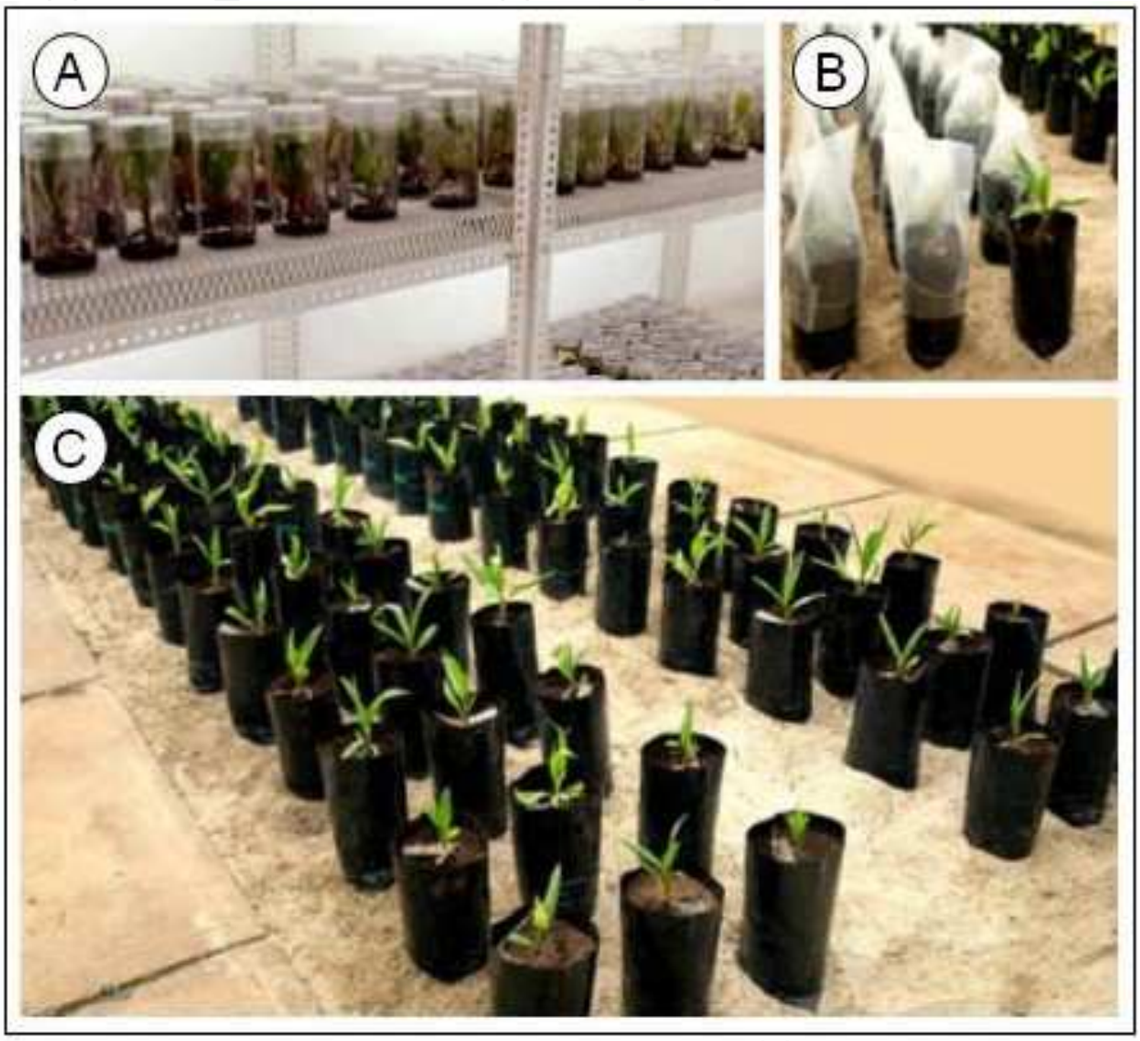

Fig. 5. Final stages of micropropagation process of coconut palm. (A) *In vitro* plantlets ready to be transferred to *ex-vitro* conditions. (B) Acclimatization of plantlets in greenhouse covered with transparent perforated bags and (C) plantlets ready to be transferred to field conditions.

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9. Abbreviations

SE: somatic embryogenesis

ZES: zygotic embryo segments.

MS: Murashige & Skoog medium (1962)

BAP, 6-benzylaminopurine

NAA: naphthaleneacetic acid

IAA: indoleacetic acid

ISE: indirect somatic embryogenesis

MS-3R: MS medium with BAP+IAA+NAA

DsRFP: Red fluorescent protein

GUS: β-glucuronidase (gene *uidA*)

pH: hydrogen potential

2, 4-D: 2, 4-dichlorophenoxyacetic acid

GA_3: gibberellic acid

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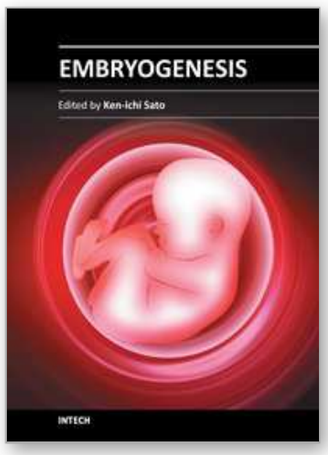

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