Chapter 16
Somatic Embryogenesis in *Agave* spp.

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Abstract The genus *Agave* is a monocotyledonous group of species that belong to the Asparagaceae family. Because of its CAM metabolism and other botanical features, the genus *Agave* is gaining importance throughout the world to address the challenges that climate change is imposing with regard to food, medicine, and bioenergy. On the other hand, it is important to point that in order to develop protocols and methods for somatic embryogenesis in species of this genus, the knowledge of its counterpart, the natural zygotic embryogenesis is crucial. Methodologies for the production of somatic embryos in this genus have been reported for *A. victoria-reginae, A. sisalana, A. salmiana, A. tequilana, A. angustifolia, A. vera-cruz, A. fourcroydes,* and *A. sisalana*; and the uni- and multicellular origin of the somatic embryos is a key characteristic that should be taken into account for special purposes and uses. The importance of culture medium, plant growth regulators, genotype, and special conditions for culture incubation will be discussed.

16.1 The Genus *Agave*

This genus conforms a group of plant species of the Asparagaceae family (formerly Agavaceae) that belongs to the monocot class of angiosperms (APG III 2009). Nowadays, the genus *Agave* is distributed in the tropical and subtropical areas of the world and represents a large group of succulent plants, and its center of origin is probably limited to México (Gentry 1982). The genus *Agave* has about 200 species of which approximately 150 are endemic to México (García-Mendoza 2002), and it is divided into two subgenera, *Littaea* and *Agave,* based on the architecture of the inflorescence; subgenus *Littaea* has a spicate or racemose inflorescence while plants

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of the subgenus *Agave* bear a paniculate inflorescence with flowers in umbellate clusters on lateral branches (Gentry 1972).

Recent studies have found that the genus *Agave* is a young genus, which is between 7.8 and 10.1 million years old (Good-Avila et al. 2006). The subgenus *Agave* and particularly the sections Rigidae and Sisalanae are cultivated because of their commercial importance for diverse purposes: (a) alcoholic beverages such as tequila and mezcal; (b) natural long and hard fibers; and (c) sapogenins as natural precursors of steroidal compounds and medicinal principles as those species of the Amolae group (Blunden et al. 1980; Gentry 1982; Cedeño 1995); and unarmed species lacking spines are frequently used as ornamental plants among many other uses. *Agave tequilana* Weber var. Azul, which is the raw material for the production of tequila is the most extended species in plantations with about 100,000 ha in the region of the appellation of origin "tequila" in México (CRT 2015). Today, the cultivation of this species involves a high degree of mechanization and the use of modern agronomical inputs with a high degree of success in the production (Valenzuela 2010). However, the cultivation of other *Agave* species used in México for the elaboration of diverse products such as mezcal, in a majority of cases still being produced under ancient practices.

Besides their economic importance for the production of alcoholic beverages and fibers, agaves are becoming key plant species for the pharmaceutical industry and to tackle climate change in the near future for the production of biofuels because of their rusticity and because they do not compete with food crops.

In general, wild and cultivated species of *Agave* perform well in areas where rainfall is not sufficient for many cultivated C_3 and C_4 plants; this is because their crassulacean acid metabolism (CAM) allows them to tolerate dry and hot environments by opening the stomata at night for CO_2 uptake, thus avoiding loss of water. This CAM photosynthetic pathway allows that most agave species may have higher productivity in areas of prolonged droughts and with water restrictions than many other plant species (Kant 2010). Escamilla-Treviño (2012) made a detailed analysis of biomass productivity with regard to drought tolerance according to approximate rainfall requirements and based on the reports of several authors. He found that *Agave* species such as *A. salmiana, A. mapisaga, A. deserti, A. fourcroydes,* and *A. tequilana* have a higher degree of tolerance to drought as compared to *Panicum virgatum* var. Alamo, *Zea mays* (grain and stover), *Populus* spp. *Miscanthus giganteus, Saccharum officinarum,* and *Sorghum bicolor.*

Because of the above characteristics, agaves have emerged as a potential solution for the production of biofuels for the reduction of greenhouse emissions. The countries that have ratified the Kyoto Protocol are committed to fulfilling the commandments of the Clean Development Mechanism, whose distinctive element of the Kyoto Protocol is its demand that countries must reduce their greenhouse gas emissions (UNFCCC 1998). Furthermore, some *Agave* species have proven to be low recalcitrant lignocellulosic feedstock for biofuels when compared to non-agave plants (Li et al. 2014). Lignin together with hemicellulose and cellulose are the principal elements of plant cell walls. For the purpose of biofuel production, lignin hinders the hydrolysis of the polysaccharides to convert the lignocellulosic mass to

biofuel making some plant species highly recalcitrant (Escamilla-Treviño 2012). Recently, it has been reported that *A. americana* leaves, *A. salmiana* leaves, *A. tequilana* leaves, and *A. americana* stem have 8.2, 9.8, 11.9, and 7.3 g/100 g biomass based on oven-dried material, respectively, while Poplar and Switchgrass have a lignin content of 23.4 and 18.8 g /100 g biomass, respectively (Li et al. 2014). Thus, some estimated theoretical maximum ethanol yields for *A. americana*, Poplar, and Switchgrass are from 963 to 3,273, 1,273, and 1,403 gallons /ha year, respectively (Li et al. 2012).

It is important to note that there exist several agave landraces belonging to the *A. angustifolia* ssp. *tequilana* complex with domestication syndrome for sugars that may be useful for biofuel production in the near future (Valenzuela 2010).

On the other hand, recently the use of fructans, especially those from several agave species are gaining importance as healthy food ingredients as soluble dietary fiber and also because of their prebiotic characteristics benefiting the gastrointestinal flora of humans and some animals (López and Urías-Silvas 2007; Espinoza-Andrews and Urías-Silvas 2012). Agave fructans possess a particular core structure for which they have been called "agavins." This particular structure escapes from the action of digestive enzymes, thus serving as substrates (prebiotics) for the microflora living in the colon (López and Urías-Silvas 2007; Velázquez-Martínez et al. 2014).

16.2 Zygotic Embryogenesis in *Agave tequilana*

The somatic embryogenesis process cannot be understood without extensive knowledge of the zygotic embryogenesis in the plant. The formation of the embryo sac and subsequent double fertilization and the early development of the embryo and endosperm have recently been studied in *Agave tequilana* Weber var. Azul. This study was carried on clarified mature and immature ovules without cutting the tissues with a microtome in order to maintain the cells in their original site inside the embryo sac. In short, the female gametophyte originates from a single haploid cell originated by the meiotic division of a megaspore mother cell. This, in turn, undergoes three mitotic divisions that occur in a synchronized way at both extremes of the embryo sac giving rise to an eight-nucleated embryo sac. In this study, it was corroborated that the mature embryo sac is of the monosporic Polygonum-type and at this stage is already cellularized and consists of seven cells: three antipodal cells located at the chalazal pole, the central cell formed by two polar nuclei located just below the antipodals, and the egg apparatus located at the micropylar pole and composed of one egg cell and two synergids (González-Gutiérrez et al. 2014). The keynote is that all structures and cells studied were highly polarized and aligned to the micropylar-chalazal axis. In this manner, the development of the embryo sac, the egg cell, the zygote, and the early embryo were polarized as in most of the angiosperms (Huang and Sheridan 1994; Dodeman et al. 1997; Sundaresan and Alandete-Saez 2010). The polarity of the egg cell and the zygote is evident from the position of the nucleus located toward the

cytoplasm-rich chalazal extreme while the micropylar pole is highly vacuolated (see Figs. 4d, 5a and additional file 2 Fig. S5 in González-Gutiérrez et al. 2014). At 6 days after pollination (DAP), the zygote elongates about 50 % its original size. Finally, at nine DAP the zygote suffers a first asymmetric cell division giving rise to a two-celled proembryo consisting of cells with different developmental fates; the basal cell that will form the suspensor and the apical cell which is the first cell of the embryo proper and that through a series of coordinated cell divisions will form the embryo. This observed process was similar to what is described for the majority of angiosperms (Lau et al. 2012; González-Gutiérrez et al. 2014; Leljak-Levanić et al. 2015). Furthermore, the same pattern has been observed in another genus member of the Asparagaceae family (formerly Agavaceae): *Polianthes tuberosa* (González-Gutiérrez, to be published elsewhere).

16.3 Zygotic Embryogenesis Versus Somatic Embryogenesis in *Agave tequilana*

Somatic embryogenesis in plants is intrinsically linked to zygotic embryogenesis. Early somatic embryogenesis stages resemble those of zygotic embryogenesis, and many phenotypic and molecular features are shared between both types of embryogenesis (Jin et al. 2014). In *A. tequilana,* the rare occurrence of dicotyledonar zygotic embryos was recently reported (Ayala-González et al. 2014). *A. tequilana* is a plant species of the Asparagaceae family that belongs to the monocot class of angiosperms. Therefore, it should contain only one cotyledon. From a total of 1,164 analyzed embryos, 4 % showed two cotyledons (or dicotyledonar embryos), 44 % showed two fused cotyledons, and 52 % showed only one cotyledon. This means that about 50 % of the analyzed embryos were of a kind of dicotyledonous nature. It is possible that PIN proteins and adjacent genetic elements are being expressed as in dicots such as *Arabidopsis thaliana* (Jenik et al. 2007).

It is considered that monocots must have evolved from a primitive dicot. If a monocotyledon is derived from a dicotyledon, it must have happened through the process known as syndactyly (Bancroft 1914). Syndactyly is a concept used for the description of the fusion of two cotyledons to form one member (Sargant 1903; Bancroft 1914; Socoloff et al. 2014). Furthermore, recently dicotyledonar somatic embryos have appeared in some genetic lines of *A. tequilana* (unpublished results). Histological sections of embryogenic cell cultures showed the early formation of somatic dicotyledonar embryos (Fig. 16.1). Most of the embryos showed two fused cotyledons and after germination, they reached the form of a normal seedling. It seems that this phenotypic trait is the expression of a genetic nature and not due to particular environmental conditions of the in vitro culture, thus being this an example of shared common characteristics between zygotic embryogenesis and somatic embryogenesis. Other cytological characters resembling those of the zygotic embryos of this species will be discussed below in the corresponding section.

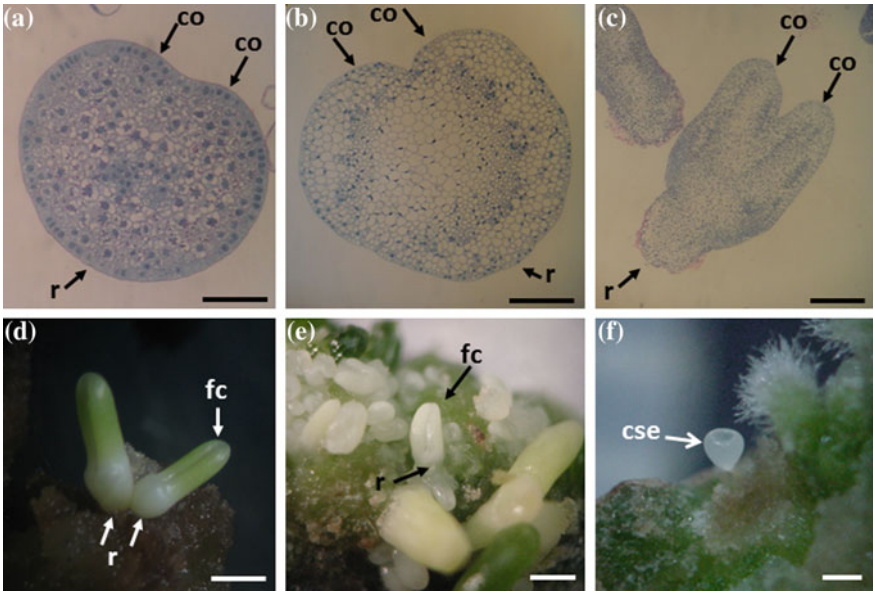

Fig. 16.1 Dicotyledonar somatic embryos of *Agave tequilana* Weber var. Azul. **a**–**b** Histology of early somatic embryos at the heart stage. Bar = 75 µm. **c** Mature somatic embryo with two cotyledons. Bar = 350 µm. **d**–**e** Somatic embryos with fused cotyledons. Bar = 1 mm. **f** *Cup-shaped* somatic embryo. Bar = 0.5 mm. *co* cotyledon; *r* radicle; *fc* fused cotyledon; *cse* cup-shaped somatic embryo

16.4 Somatic Embryogenesis in *Agave* spp.

For ease of time and space, in this revision, only basal media and growth regulators of the revised protocols will be mentioned, and some particular procedures and materials will be discussed where applicable. In this context, Table 16.1 summarizes general aspects of explant and medium composition for the somatic embryogenesis of several agave species.

16.4.1 Agave victoria-reginae

The first report on the somatic embryogenesis in the genus *Agave* was on the ornamental species *A. victoria-reginae* (Rodríguez-Garay et al. 1996). Direct somatic embryos were produced from young leaf blades harvested from in vitro propagated plantlets. The induction medium consisted of MS medium (Murashige and Skoog 1962) supplemented with 0.3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Embryo germination was achieved by transferring globular embryos to growth regulator-free half strength MS medium; however, the germinated embryos

Table 16.1 Plant growth regulators used for induction of somatic embryogenesis in *Agave* spp.

Agave species	Explant	Response	Medium + PGR mgL^{-1}	References
victoria-reginae	In vitro leaf blades	Direct SE	MS + 0.3 2,4-D	Rodríguez-Garay et al. (1996)
sisalana	In vitro stems	Indirect SE	MS + 0.25 2,4-D + 1.0 BAP	Nikam et al. (2003)
victoria-reginae	Seedling stem segments	Indirect SE	MS + 0.52 2,4-D	Martínez-Palacios et al. (2003)
salmiana	In vitro leaf blades	Indirect SE	MS + 0.5 NAA + 1.1 BAP	Flores-Benítez et al. (2007)
tequilana	In vitro leaf blades	Indirect SE	MS + 2.0 2,4-D + 0.3 BAP	Portillo et al. (2007)
vera-cruz	In vitro leaf blades	Indirect SE	MS + 1.0 NAA + 0.2 ZEA	Tejavathi et al. (2007)
angustifolia	Zygotic embryo	Indirect SE	MS + 3.0 2,4-D + 1.0 BAP	Arzate-Fernández and Mejía-Franco (2011)
tequilana	In vitro leaf blades	Indirect SE	MS + 3.0 2,4-D + 0.3 BAP SH + 3.0 2,4-D + 0.3 BAP	Rodríguez-Sahagún et al. (2011)
fourcroydes	In vitro stems	Direct SE	MS + 0.5 DIC MS + 0.5 PIC	Monja-Mio and Robert (2013)
sisalana	Bulbils	Indirect SE	MS + 3.0 2,4-D + 20.0 BAP	Carneiro et al. (2014)

PGR Plant Growth Regulators; *SE* Somatic embryogenesis; *2,4-D* 2,4-dichlorophenoxyacetic acid; *BAP* 6-benzylaminopurine; *NAA* naphthaleneacetic acid; *ZEA* zeatin; *DIC* dicamba; *PIC* picloram; *MS* Murashige and Skoog (1962); *SH* Schenk and Hildebrandt (1972)

became hyperhydric. Hyperhydricity was completely eliminated by the use of vented Petri dishes, where the vents were covered with filter paper to facilitate gas exchange and MS medium with the concentration of NH_4NO_3 reduced to 5 mM. Plantlets from somatic embryos resulted habituated for growth regulators and at present are still propagated by shoot proliferation in completely hormone-free MS medium. Finally, the adaptation of several hundreds of plants to their natural habitat was successful. Moreover, Martínez-Palacios et al. (2003) successfully produced indirect somatic embryos from seedling stem segments in the same species by the addition of 0.5 mgL^{-1} 2,4-D to MS medium. These authors claimed a multicellular origin of the somatic embryos.

16.4.2 Agave sisalana

Agave sisalana (also known as Sisal) is a cultivated pentaploid species ($2n = 5x = 150$) (Castorena-Sánchez et al. 1991), which is used in many countries for the extraction of fibers from leaves and also for the secondary metabolites of pharmacological importance (Nikam et al. 2003; Debnath et al. 2010; Carneiro et al. 2014).

The first report of somatic embryos in this species is that of Nikam et al. (2003). They found that under a prolonged culture of 5–7 weeks in MS medium supplemented with 1–2 mg L^{-1} kinetin (KIN) or 0.25–0.5 mg L^{-1} naphthaleneacetic acid (NAA) + 1–1.5 mg L^{-1} KIN or 6-benzyladenine (BA), new embryos developed from embryogenic callus. However, the most effective medium for the induction of somatic embryos was supplemented with 0.25 mg L^{-1} 2,4-D, in which the embryogenic potential was maintained for about 48 months. In this protocol, MS medium + 0.1 or 0.2 mg L^{-1} KIN were used for embryo expression and germination which was achieved in 5 weeks. Histological analyzes of somatic embryogenesis showed that both unicellular and multicellular processes were the origin of somatic embryos.

In a more recent study on the somatic embryogenesis of *A. sisalana* conducted by Carneiro (2014), the best culture medium was half the concentration of MS salts supplemented with 3.0 mg L^{-1} 2,4-D + 20.0 mg L^{-1} BA. The cytological and histological analyzes of embryogenic cultures showed a clear unicellular origin as it has been found in other studies conducted in *A. tequilana* (Gutiérrez-Mora et al. 2004; Portillo et al. 2007; Santacruz-Ruvalcaba and Portillo 2009).

16.4.3 Agave salmiana

This species is cultivated in several regions of México and is used for the alcoholic beverages pulque and mezcal. Also, it is widely used for ethnomedical purposes and fodder in desert lands (Colunga-GarcíaMarín et al. 2007; Flores-Benítez et al. 2007). The somatic embryogenesis was achieved on a study about the genetic transformation of the species by using leaf blades from in vitro-produced plantlets. Somatic embryos were produced on MS medium with the addition of 0.5 mg L^{-1} NAA and 1.1 mg L^{-1} BA, and supplemented with a mixture of vitamins and amino acids reported by Mere-Villanueva and Vázquez-Alejandro (2003) that consisted of 306.38 μM glycine, 804.84 μM myoinositol, 12.18 μM nicotinic acid, 7.30 μM pyridoxine HCl, 8.90 μM thiamine HCl, 66.62 μM L-asparagine, 4.10 μM biotin, 57.40 μM L-arginine, 56.35 μM L-aspartic acid, 410.67 μM glutamine, 51.0 μM glutamic acid, 2.26 μM folic acid, 0.26 μM riboflavine and 749.25 μM urea (Flores-Benítez et al. 2007). Finally, in this work, *A. salmiana* transformed plants were regenerated from embryogenic callus co-cultivated with *Agrobacterium tumefaciens.*

16.4.4 Agave tequilana

As stated above, *A. tequilana* is the most widely cultivated species of agave in México with about 100,000 ha. Since the 1990s, this species has been severely attacked by diverse diseases caused by bacteria and fungi, and exposed to a multitude of natural abiotic stressors, which reduce both quality and yield of fermentable juices. During the past few years, the bacterium *Erwinia carotovora* and the fungus *Fusarium oxysporum* have been causing severe damage to *A. tequilana* plantations in México, including the states of Guanajuato, Jalisco, Michoacán, Nayarit, and Tamaulipas (Jiménez-Hidalgo et al. 2004; Ávila-Miranda et al. 2010). Moreover, constant high temperatures imposed by climate change have been of strong impact on agaves. While *A. tequilana* is commercially reproduced by rhizomatous suckers for new plantations, blooming plants have shown severe abnormalities in their flowers, mainly in the female reproductive apparatus. This means that the whole plant is under stress, diminishing the possibilities of a good productivity for the tequila industry (Rodríguez-Garay et al. 2014). The previously mentioned problems have pushed researchers to find biotechnological alternatives for the micropropagation and the genetic improvement of this important species.

The first protocol for the somatic embryogenesis in *A. tequilana* was reported by Portillo et al. (2007). Somatic embryos were produced from leaf blades collected from six in vitro micropropagated genotypes and cultured on MS medium supplemented with L2 vitamins (Phillips and Collins 1979) with the addition of several growth regulators. In this study, it was found that for the induction of somatic embryogenesis some genotypes gave good embryo production under high cytokinin concentration and low auxin concentration while other genotypes showed a good response to relatively high auxin concentration and low cytokinin concentration. In this manner, the genotype named S3 produced somatic embryos with 10.0 and 15.0 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D. On the other hand, genotype S7 produced somatic embryos with 2.0 mg L^{-1} 2,4-D and 0.3 mg L^{-1} BA. These highly contrasting responses can only be attributed to the genotype of the mother plant. In all cases, the expression and maturation of embryos were achieved on MS medium without growth regulators and supplemented with 500 mg L^{-1} L-glutamine and 250 mg L^{-1} casein hydrolysate. Moreover, in this work the unicellular origin of the somatic embryos was demonstrated (Fig. 16.2). On the other hand, an elegant demonstration of the unicellular origin of the *A. tequilana* somatic embryos was reported by Rodríguez-Domínguez (2000). In this study, gamma rays were used for the bombardment of highly embryogenic cells causing a mutation of the apical cell that resulted from a first cell division giving rise to an albino plantlet with green radicle.

In general terms, the initial embryonic cell is immersed in a proembryogenic cell mass and emulates the zygote which is its zygotic equivalent; its polarity is evident and contains large amounts of starch granules (Fig. 16.2a, b). The first and second divisions are highly polarized and start to show the first suspensor cells (Fig. 16.2c, d). After some rounds of cell divisions, the somatic embryo shows initials of a

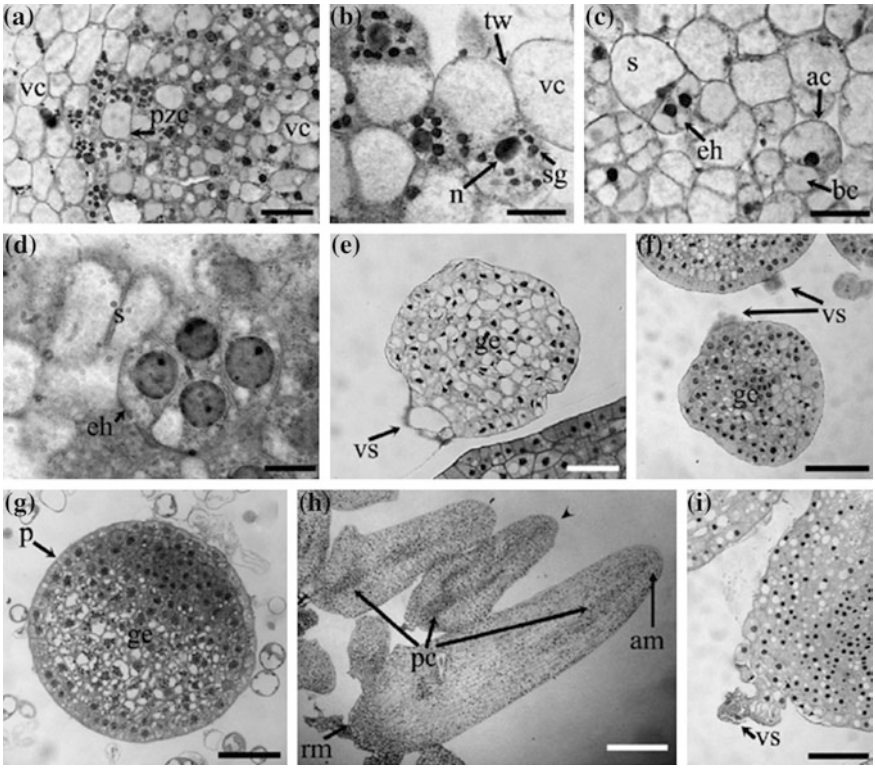

Fig. 16.2 Early stages of somatic embryogenesis in *A. tequilana* Weber var Azul. **a** Callus formed by highly vacuolated noncompetent cells and embryogenic cells containing large amounts of starch granules. Bar = 40 μm. **b** Polarized embryogenic cells containing large amounts of starch granules. Bar = 20 μm. **c** Polarized embryogenic structures resulting from first and second division of embryogenic cells. Bar = 30 μm. **d** Four-celled proembryo with a suspensor-like structure. Bar = 10 μm. **e** and **f** Globular embryos with vestigial suspensor. Bars = 50 and 100 μm. **g** Globular somatic embryo without suspensor. Bar = 75 μm. **h** Torpedo stage embryos showing procambial initials. Bar = 350 μm. **i** Close-up of the vestigial suspensor of one of the embryos in (*h* arrow head). Bar = 20 μm. *Pzc* polarized cell; *vc* vacuolated cell; *sg* starch grain; *n* nucleus; *tw* thick wall; *eh* embryo head; *s* suspensor; *ac* apical cell; *bc* basal cell; *ge* globular embryo; *vs* vestigial suspensor; *p* protoderm; *pc* procambial strands; *rm* root meristem; *am* apical meristem. From Portillo et al. (2007) Somatic embryogenesis in *Agave tequilana* Weber cultivar Azul. In Vitro Cell Dev-Pl 43:569-575. Copyright 2007 by the Society for In Vitro Biology, formerly the Tissue Culture Association. Reproduced with permission of the copyright owner

remaining suspensor and reaches its globular stage with an evident and well-formed protoderm (Fig. 16.2e, f, g). The embryos in the torpedo stage show the procambial initials and are ready for germination (Fig. 16.2h). At this point, it is important to mention that this cytological and morphological characteristic (suspensor) in the somatic embryo, is initiated from the basal cell of the very first division of the embryogenic cell; in the somatic embryo this is a key point for the formation of the

radicle and the final polarity of the new plant (Gutiérrez-Mora et al. 2012). On the other hand, it has been demonstrated that the medium basal composition plays an important role in the success of somatic embryogenesis in *A. tequilana*. When SH medium (Schenk and Hildebrandt 1972) was used instead of the MS medium, the production of somatic embryos was highly reduced. It is possible that the higher concentration of some ions in the MS medium is responsible for this effectivity. Furthermore, in this work, it was found that light quality exerts an important effect on the induction, maturation, and germination of the agave somatic embryos. Blue light produced a high number of embryos (an average of 20 per explant). However, the production of embryos increased when the white or red light was used for the induction period and then wide-spectrum light for the expression and maturation phase (Rodríguez-Sahagún et al. 2011).

Moreover, it is known that arabinogalactan proteins (AGPs) exert an important control on zygotic and somatic embryogenesis by stimulating both processes (Samaj et al. 2006). Recently, a study was conducted to investigate the distribution of AGPs and pectin in *A. tequilana* by using immunolabeling with anti-AGP monoclonal antibodies JIM4, JIM8 and JIM13 and anti-methyl-esterified pectin-antibody JIM7. Besides the presence of starch granules, it was found that AGPs and pectin are directly related to the embryogenic capability of somatic cells. These findings may be useful for selecting embryogenic genotypes, providing a new tool for the optimization of the somatic embryogenesis process (Portillo et al. 2012).

In regard to genetic improvement, somatic embryogenesis protocols have been used to produce trisomic, triploid and haploid plants. This goal was achieved with induction of trisomy by exposing embryogenic cells to 8 mg L^{-1} *para*-fluorophenylalanine (PFP) added to the induction medium. Obtained plants were trisomic with $2n = 2x = 61$; and there were differences in chromosome arm ratio (long arm/short arm) in eight chromosome pairs and more than 13 homologous chromosome pairs exhibited structural changes; all these aberrations in the chromosome complement of trisomic plants were putatively caused by inversions, deletions, and/or duplications produced by high concentrations of PFP; and the presence of a single extra chromosome could have been induced by the effect of PFP on the mitotic spindle by inducing nondisjunction of sister chromatids, resulting in cells with $2n + x$ and $2n - x$ chromosomes. In vitro-produced plants were transferred to soil and have continued to grow under ex vitro conditions. Trisomic plants showed remarkable morphological characteristics, such as longer terminal spines and wider leaves, as compared as to wild-type or normal plants (Ruvalcaba-Ruíz et al. 2012). Moreover, triploid plants have been regenerated from somatic embryos produced from the immature triploid endosperm of *A. tequilana*. Age (45 days after pollination), the genotype of the parents, growth regulators and light quality played important roles in the production of triploid embryos. Two embryogenic calluses were obtained by culture on *N* medium (Nitsch and Nitsch 1969) and MS medium with the addition of 2 mg L^{-1} 2,4-D, and 0.3 mgL^{-1} BA. After the induction period, embryogenic calluses were transferred to LOG medium (Castro-Concha et al. 1990) without growth regulators and supplemented with 4 mg

L^{-1} BA and exposed to red light (λ = *630 nm) for 15 days. It was claimed that red light was a key element for the regeneration of triploid plants from the endosperm. These two calluses regenerated two plants that had triploid cells with 90 chromosomes (Ruvalcaba-Ruíz 2003). On the other hand, haploid plants are important individuals in plant breeding programs for a vast number of genetic methodologies, such as the production of completely homozygous plants and the selection of recessive traits among many other uses. Ruvalcaba-Ruíz (2003) produced a haploid plant by culturing unpollinated ovaries of* A. tequilana *on NPB medium supplemented with 90 gL^{-1} maltose, 300 mgL^{-1} casein hydrolysate, 2.0 mgL^{-1} 2,4-D and 0.3 mgL^{-1} BAP for the induction of somatic embryogenesis. Embryogenic callus was transferred to MS medium with the addition of 4.0 mg L^{-1} BA and incubated for 15 days under red light (λ = 630 nm). A plant was obtained from a regenerated somatic embryo which was found to be haploid with 30 chromosomes.*

Current work is focused on the application of several strategies with the development of cell and tissue culture methodologies as well as in casa hybridization techniques, which include embryo rescue (to be published elsewhere) in order to be used in the genetic improvement of this important industrial agave species.

16.4.5 Agave angustifolia

This species is widespread all over México and cultivated in many countries. In México, one of the most important uses is for mezcal production among other industrial and medicinal purposes. The somatic embryogenesis in *Agave angustifolia* was recently achieved by the use of zygotic embryos as explants. These embryos were cultured for the induction process on 25 % MS medium supplemented with 3.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} BA, and 60 g L^{-1} sucrose and incubated under dark conditions. The expression and germination medium consisted of half strength MS medium without growth regulators. Regenerated plants were obtained 140 days after the beginning of the in vitro culture (Arzate-Fernández and Mejía-Franco 2011). It is well known that diverse kinds of stresses and plant growth regulators play an important role in somatic embryogenesis. The utilization of 25 % MS medium and the addition of 60 g L^{-1} sucrose in the induction process could have acted positively as starvation and osmotic stresses, respectively, as it has been demonstrated in other plant species (Jin et al. 2014).

16.4.6 Agave vera-cruz

This species is an unknown plant in México. *A. vera-cruz* is cultivated in some regions of South India for fiber, food, and medicine and it is known as "Grey Aloe of India" (Tejavathi et al. 2007); and it has been studied and well characterized

since the early 1950s in India as a source of carbohydrates (Srinivasan and Bratia 1953; Cairns 1993).

Shoot apices, cotyledons, and leaf segments from 3 months old seedlings were used as explants. Diverse vitamin compositions were tested, finally for all somatic embryogenesis experiments L2 vitamins (Phillips and Collins 1979) were chosen because of its ease of rapid production of callus.

Embryogenic callus was induced on MS medium supplemented with 1.0 mg L^{-1} NAA and 0.2 mg L^{-1} zeatin (ZEA) with the addition of 40 g L^{-1} sucrose. Expression and germination of somatic embryos were achieved in the same MS medium.

Rooted plantlets were transferred to soil with a survival of 96–98 % without any hardening procedure. The authors claimed that the origin of somatic embryos was of a multicellular kind (Tejavathi et al. 2007).

16.4.7 Agave fourcroydes

This *Agave* species known as "henequén" is well adapted to the arid areas of México and Central America including Caribbean countries such as Cuba. *A. fourcroydes* is a pentaploid, long-lived plant, asexually propagated and grown mainly for the manufacture of ropes, woven sacks of high quality, and for the extraction of medicinal precursors (Gentry 1982). Recently, Monja-Mio and Robert (2013) reported the direct somatic embryogenesis in this species through thin cell layer culture (tTCLs), which will be a useful biotechnological technique for in vitro germplasm conservation, genetic improvement and for micropropagation as it has been reported for many plant species. For this purpose, thin tissue segments (tTCLs) of 0.5–1.0 mm from stems taken from in vitro propagated plantlets were used as explants. Induction of somatic embryogenesis was achieved by culturing the explants on MS medium supplemented with L2 vitamins (Phillips and Collins 1979), 0.5 mg L^{-1} dicamba (DIC) or 0.5 mg L^{-1} picloram (PIC), 30 g L^{-1} sucrose, solidified with 3 g L^{-1} agar and 3 g L^{-1} Phytagel and incubated under dark conditions. The embryogenic response was improved when the explant donor plantlets were maintained for one month on a culture medium containing 10 mg L^{-1} BA. Again in this agave species, the embryogenic response was strongly dependent on the genotype. Somatic embryos did not show any vascular connection with the original explant tissue and seemed to be generated through uni- and multicellular events. These embryos germinated when they were transferred to half strength MS medium and regenerated plantlets were transferred to soil and maintained under greenhouse conditions with a survival rate of 85 %.

16.5 Concluding Remarks

Somatic embryogenesis has been achieved in several species of the genus *Agave*. Scientific reports have indicated that the production of somatic embryos is feasible in species from both subgenera, *Littaea* and *Agave* such as *A. victoria-reginae* and *A. tequilana,* respectively. At this point, it is important to remark that more knowledge in depth is necessary about zygotic embryogenesis in order to understand the cytological, biochemical, and molecular mechanisms for developing protocols for somatic embryogenesis; being this one of the most important biotechnological tools for conservation, micropropagation, and the genetic improvement of *Agave* species of ecological and economic importance.

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