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Review

Tissue culture of ornamental cacti

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ABSTRACT: Cacti species are plants that are well adapted to growing in arid and semiarid regions where the main problem is water availability. Cacti have developed a series of adaptations to cope with water scarcity, such as reduced leaf surface via morphological modifications including spines, cereous cuticles, extended root systems and stem tissue modifications to increase water storage, and crassulacean acid metabolism to reduce transpiration and water loss. Furthermore, seeds of these plants very often exhibit dormancy, a phenomenon that helps to prevent germination when the availability of water is reduced. In general, cactus species exhibit a low growth rate that makes their rapid propagation difficult. Cacti are much appreciated as ornamental plants due to their great variety and diversity of forms and their beautiful short-life flowers; however, due to difficulties in propagating them rapidly to meet market demand, they are very often over-collected in their natural habitats, which leads to numerous species being threatened, endangered or becoming extinct. Therefore, plant tissue culture techniques may facilitate their propagation over a shorter time period than conventional techniques used for commercial purposes; or may help to recover populations of endangered or threatened species for their re-introduction in the wild; or may also be of value to the preservation and conservation of the genetic resources of this important family. Herein we present the state-of-the-art of tissue culture techniques used for ornamental cacti and selected suggestions for solving a number of the problems faced by members of the Cactaceae family.
Keywords: Cactaceae, areole activation, conservation, micropropagation, ornamental cactus

Introduction

The Cactaceae family is native to the American continent and comprises more than 2,000 species that are primarily distributed over four diversity centers in arid and semiarid regions. The most important centers of cactus diversity are the north-central region of México through to the southwest of the United States, known as the Chihuahuan Desert Ecoregion (CDE), and the arid and semiarid zone of the southwestern Andean region. The latter includes parts of Peru, Chile and Argentina. Other areas with high diversities of cacti are eastern Brazil, the region of Central America and part of southeastern México, where a significant group of humid-zone epiphytic species is distributed (Hernández and Bárcenas, 1995, 1996; Ortega-Baes and Godínez-Álvarez, 2006).

México, Argentina, Peru, Bolivia, Chile and Costa Rica have the highest proportion of endemic species. México, with more than 600 species, of which approximately 80 % are endemic, is the most important center of concentrated cactus genera and species (Ortega-Baes et al., 2010). In México, two regions are particularly rich in species with high degrees of endemism: the southeastern and eastern regions of the CDE and the Querétaro-Hidalgo Arid Zone (QHAZ) (Hernández and Bárcenas, 1995, 1996). Other important centers of high cacti diversity in México are the Sonoran Desert (Turner et al., 1995), the Tehuacán-Cuicatlán Valley and the Balsas River Basin at the Tehuantepec Isthmus. Significant relationships have been observed between species richness and endemism and species richness and number of endangered species (Godínez-Álvarez and Ortega-Baes, 2007).

Brazil is the third cactus diversity center in importance in the American continent with a high degree of endemism (Zappi et al., 2011). Cactus species distribution and the number of endemic species (in brackets) in Brazilian regions is as follows: northeastern region 90 (34), southeastern 120 (100), center-western 33 (6) southern 70 (41) and northern 17 (1) (Zappi et al., 2011). Native cactus genus and species-subspecies include *Arrojadoa* (8), *Arthrocereus* (7), *Bragaia* (1), *Brasilicereus* (2), *Brasiliopuntia* (1), *Cereus* (20), *Cipocereus* (8), *Coleocephalocereus* (11), *Discocactus* (14), *Echinopsis* (4), *Epiphyllum* (1), *Espostoopsis* (1), *Estevesia* (1), *Facheiroa* (5), *Frailea* (17), *Gymnocalycium* (8), *Harrisia* (2), *Hatiora* (3), *Hylocereus* (1), *Leocereus* (1), *Lepismium* (4), *Melocactus* (31), *Micranthocereus* (12), *Opuntia* (6), *Parodia* (36), *Pereskia* (9), *Pilosocereus* (45), *Praecereus* (1), *Pseudorhipsalis* (1), *Quiabentia* (1), *Rhipsalis* (42), *Schlumbergera* (11), *Stephanocereus* (2), *Strophocactus* (1), *Tacinga* (10) and *Uebelmannia* (8) (Zappi et al., 2011). Uses and knowledge of cacti species in northeastern Brazil have been summarized by Lucena et al. (2013).

Cacti are perennial plants, succulent and slow-growing, which are particularly known for their drought-tolerant characteristics (xerophytic). They are highly prized by horticulturists as botanical oddities and ornamental plants. They may be cultivated for their beautiful flowers, the aesthetics of their stems and spines, or mere-

ly because the plants have an original-look/unique morphology. Individual cactus species may be arborescent, shrubby or creeping with woody or succulent stems. The latter may be globular, cylindrical, candelabrum, columnar, oblong or cladode in shape, with spines distributed uniformly around the stem or forming longitudinal ribs (Nobel, 1988). The tallest cactus is *Pachycereus pringlei* (S. Watson) Britton & Rose, with a recorded maximum height of 19.2 m, and the smallest is *Blossfeldia liliputiana* Werdermann, which is approximately 1 cm in diameter at maturity (Altesor and Ezcurra, 2003).

Cactus spines are produced by specialized structures called areoles, a type of meristematic tissue. Areoles are an identifying feature of cacti. In addition to spines, areoles produce new branches and flowers, which are typically tubular and multipetal. Cactus flowers are very attractive and, depending on the species, are diverse in size, number, form and color. The nocturnal flowers are always white, with some light-yellow or red tones, whereas diurnal flowers are white, purple, yellow-orange, red or green (Barthlott and Hunt, 1993; Anderson, 2001). Cacti are particularly attractive to homeowners in arid regions where conserving water by substituting drought-tolerant plants for water-intensive vegetation (such as grass lawns) is increasingly encouraged.

Cacti have been cultivated as ornamental plants for centuries. Since the XVI century, several herbariums (General Historie of Plants, 1597; Pinax Theatri Botanici, 1623; Historia Plantarum, 1688; History of Succulent Plants, 1718, among others) and botanic gardens in Europe have established collections of cacti and succulents (Pizzeti, 1992). Cacti are very often grown in greenhouses, particularly in regions unsuited to outdoor cultivation such as the northern parts of Europe and North America. They are cultivated in pots or grown in the ground. Cacti are also grown as houseplants, with many being tolerant to the often-dry atmosphere and are maintained under cover during the winter. Cacti may also be planted outdoors in regions with suitable climates. These plants are widely used as ornamentals worldwide.

In the USA, cacti are very common as ornamentals in Arizona and Nevada and are found to varying degrees in California, New Mexico and Texas. The genera *Mammillaria* and *Opuntia* [*O. phaecantha* Engelm., *O. engelmannii* (Griffiths) A. Nelson, *O. violacea* Engelm., *O. basilaris* Engelm. & Bigelow and *O. ficus-indica* (L.) Mill.] are the most commonly observed ornamentals. Other common species include *Schlumbergera truncata* (Haworth), known as Christmas cactus or thanksgiving cactus, a flat-stemmed, red-, pink-, or white-flowered species that is grown as a garden or houseplant; the candelabra cactus (*Cereus peruvianus* Engelm. & Bigelow), a tree-sized species native to South America (Anderson, 2001; Irish, 2001). The genera *Hatiora* and *Schlumbergera* are economically important glasshouse crops in northern Europe, where they are cultivated widely as flowering potted plants. *Hatiora gaertneri* (Regel) Barthlott, *H. rosea* (Lagerh.) Barthlott, and their interspecific hybrid (= *H.* × *graeseri* Barthlott ex D.R. Hunt) are commonly known as Easter or holiday cacti.

Based on an international review of cactus markets, the United States is the leading market for CDE cactus species, with 318 species advertised by U.S. vendors and 54 identified for sale exclusively in the United States. The United Kingdom is the second-largest market for CDE species (197) followed by Germany (185), Sweden (118), México (91), Spain (86), Italy (80) and Canada (5). In the USA, west Texas is the largest cactus producer for urban markets in Arizona, Nevada and southern California. Over the past decade, cacti species endemic to México that were new to science have appeared for sale in foreign advertisements suggesting that specimens (most likely seeds) had been illegally exported from México (Robbins, 2002, 2003).

Recently, Korea has emerged as an important producer of grafted cacti. Production had reached 48 million plants in 2002 with an estimated value of US$ 2.26 million and reached almost US$ 4 million in 2011. The cactus *Gymnocalycium mihanovichii* (Fric & Guerke) Britton & Rose represents 70 % of exports, *Chamaecereus silvestrii* (Speg.) Britton & Rose 20 % and *Notocactus scopa* (Spreng.) A. Berger, *Eriocactus leninghausii* (K. Sch.) Bckbg. and *Gymnocalycium baldianum* (Speg.) Speg. 10 %. The Netherlands, the USA, China and Canada are the major importing countries for grafted cacti (Myeong et al., 2004).

In the global market of CDE cactus species, 64 % of trade relates to *Mammillaria* (93 species), *Opuntia* (47 species), *Coryphanta* (37 species) and *Echinocereus* (35 species) genera. In contrast, seven genera, each with a single species, *Geohintonia mexicana* Glass & W.A. Fitz Maurice, *Hamatocactus crassihamatus* (F.A.C. Weber) Buxb., *Isolatocereus dumortier* (Scheidw.) Backeb, *Leuchtenbergia principis* Hook, *Neolloydia conoidea* (DC) Britton & Rose, *Obregonia denegrii* Fric and *Strombocactus disciformis* (DC) Britton & Rose represent only 2 % of the species involved in the international market. *Aztekium* and *Lophophora* comprise two commercial species each [*Astekium ritteri* (Boedeker) Boedeker ex A. Berger) and *A. hintonii* Glass & W.A. Fitz Mauriceand*; Lophophora williamsii* (Lemaire ex Salm-Dyck) J.M. Coulter and *L. diffusa* (Croizat) Bravo] (Bárcenas-Luna, 2003).

One method of measuring the commercial availability and popularity of ornamental cacti is the ratio of species involved in international trade divided by the number of species in a particular genus, which is called the market representation percentage (MRP). MRP measures the number of species of a genus offered in the marketplace. Thus, an MRP of 100 indicates a high demand for the species in the market, whereas a low MRP indicates low popularity among collectors. The genera *Ariocarpus*, *Astrophytum*, *Aztekium*, *Epithelantha*, *Geohintonia*, *Hamatocactus*, *Isolatocereus*, *Leuchtenbergia*, *Lophophora*, *Neolloydia*, *Obregonia*, *Pelecyphora*, *Strombocactus* and *Thelocactus* have an MRP of 100 (Bárcenas-Luna, 2003). The species of each genus that show high market demand are shown in Table 1.

Table 1 – Ornamental cacti with high demand in the international trade market and their conservation status[1].

Genus	Species	Status[2]	Collected from the wild
Ariocarpus	*A. Agavoides*	CITES I; E; Pr	No
	A. bravoanus var. *bravoanus*	CITES I; E; Id	No
	A. bravoanus var. *hintonii*	CITES I; P	No
	A. kotschoubeyanus	CITES I; Sp	No
	A. retusus	CITES I; Pr	Yes
Astrophytum	*A. asterias*	CITES I; E; ID	No
	A. capricorne	V; Th	No
	A. myriostigma	V; Th	Yes
	A. ornatum	I; Th	No
Aztekium	*A. hintonii*	R; Sp	No
	A. ritteri	CITES I; R; Th	No
Epithelantha	*E. bokei*	V; Th	No
	E. micromeris	E; Sp	No
Geohintonia	*G. mexicana*	Sp	No
Hamatocactus	*H. crassihamatus*	Th	No
Isolatocereus	*I. dumortieri*	-	No
Leuchtenbergia	*L. principis*	R; Th	No
Lophophora	*L. difussa*	R; Id	No
	L. williamsii	Sp	No
Neolloydia	*N. conoidea*	-	No
Obregonia	*O. denegrii*	CITES I; R; Th	Yes
Pelecyphora	*P. aselliformis*	CITES I; R; Sp	No
	P. strobiliformis	CITES I; V; Th	No
Strombocactus	*S. disciformis*	CITES I; Th	Yes
Thelocactus	*T. bicolor*	V; Th	Yes
	T. conothelos	V; Sp	No
	T. hastifer	V; R; Th	No
	T. heterochromus	R; Th	No
	T. hexaedrophorus	-	No
	T. lausseri	-	No
	T. leucacanthus	Sp	No
	T. macdowellii	V; Th	No
	T. rinconensis	R; Th	No
	T. setispinus	-	No
	T. tulensis	V; Th	No

[1]Adapted from Bárcenas-Luna (2003) and MEXICAN NOM 054-2010; [2]Status: CITES1: Appendix I; IUCN: V (vulnerable), R (rare), E (endangered), I (indeterminate); NOM: Id (in danger), Th (threatened), R (rare), Sp (special protection).

There are three primary markets for ornamental cacti: nurseries, supermarkets and private collectors. Nurseries supply cacti for residential gardens, commercial establishments (e.g.:, golf courses) or public areas, such as parks. Xeriscaping or landscaping with plants adapted to arid climates is gaining popularity in desert cities where water shortages are a growing concern. Due to their large showy appearance, the species most frequently used in landscaping projects are barrel cactus (*Ferocactus* spp.), prickly pear cactus (*Opuntia* spp.) and saguaro cactus [*Carnegiea gigantea* (Engelm.) Britton & Rose]. In supermarkets, large quantities and varieties of miniature cacti grown from seeds in California and Arizona are purchased as indoor ornamental plants (Robbins, 2002; Bárcenas-Luna, 2003; Bárcenas, 2006). Private collectors represent a specialized market that is primarily interested in rare or newly discovered species within the Cactaceae family with high commercial value, such as *Pelecyphora strobiliformis* (Werdermann) Fric et Scheelle, *Strombocactus disciformis* (De Candolle) Britton & Rose, *Geohintonia mexicana* Glass & W.A. Fitz Maurice, *Aztekium hintonii* Glass & W.A. Fitz Maurice and *Mammillaria luethyi* G.S. Hinton; these latter species are endemic to the state of Coahuila (Mexico) and have only recently been described by scientists (Fuller, 1985; Bárcenas-Luna, 2003; Robbins, 2003).

Cactus prices are quite variable and are primarily influenced by the size of the plants; for example, the price of a 2-cm *Ariocarpus fissuratus* (Engelm.) K. Schum. plant is US$ 3.00; however, the price increases to US$ 125 for a 10-cm plant. Similarly, in the UK the price of cacti propagated from seeds range from £ 3.00 up to £ 200 for mature plants (Bárcenas-Luna, 2003; www.dailymail.co.uk/news/article-2131625/Britains-biggest-cactus-grower-sales-spike-hosepipe-ban-takes-toll-ordinary-garden-plants.html#ixzz2PKg1R7QJ).

Conservation status of ornamental cacti

Despite having evolved beneficial adaptations that help them tolerate arid climates, cacti are among the most threatened groups of plants in the world (Hernández and Bárcenas, 1996). Approximately 48 species are listed in the Red Data Book of the International Union for the Conservation of Nature (IUCN 2009), and almost 40 species are included in Appendix I of the Convention on International Trade of Endangered Species (CITES 1990). Due to its commercial significance, the Cactaceae family is in Appendix II (Hunt, 1999; Luthy, 2001). In México, 285 species have been included in red lists of the Mexican Environmental Agency (SEMARNAT, 2010); no fewer than 115 of these native Mexican cacti were described as occurring naturally within or adjacent to the CDE (Anderson et al., 1994; Hernández and Bárcenas, 1996). The conservation status of cacti species that are in high demand in international markets is presented in Table 1.

The ecological and biological characteristics of cacti that increase their vulnerability include: i) their great habit-specificity and edaphic-specialization (Valiente-Banuet and Godínez-Álvarez, 2002; Zavala-Hurtado and Valverde, 2003); ii) their requirement for nursing, which provides protection against predators, increases nutrient availability and supplies shade for seedlings (Castro-Cepero et al., 2006; Muro-Pérez et al., 2012); iii) their slow growth rates and long reproductive periods (Nobel, 1988; Zavala-Hurtado and Díaz-Solis, 1995; Tufenkian, 1999); iv) their low seed production, seed dormancy and low-vigor seeds greatly reduce cacti density (Mandujano et al., 2005; Flores et al., 2008); and v) self-incompatibility (Boyle and Idnurm, 2001).

In the wild, devastation of their natural habit and over-collecting reduces the cactus population. The construction of a dam near Zimapan, México, caused the

destruction of a large part of the natural habitat of *Echinocactus grusonii* Hildm. Urban development and construction of highways have altered or destroyed cactus habitats in several regions of México, New Mexico and Arizona including the Sonoran Desert. The conversion of land to agriculture has affected populations of *Ariocarpus kotschoubeyanus* (Lem.) K. Schum. in México, where dry plains were plowed for maize cultivation, and of *Copiapoa* and *Eulychnia* in Chile, where valley slopes were planted with vines (*Vitis vinifera* L.). The natural habit of *Turbinicarpus pseudomacrochele* (Backeb.) Buxb. & Backeb. and *A. kotschoubeyanus* in the desert area of Querétaro state has been severely reduced due to open mining, and in the Huizache region at San Luis Potosí, a similar situation is currently developing with a subpopulation of *T. schmiedickeanus* (Boed.) Buxb. & Backeb. (Hernández et al., 2001; Sotomayor et al., 2004). In many areas, grazing by introducing animals, such as goats, has caused serious damage to populations of cacti in North America, the Galapagos Islands and has led to a critical reduction of *Browningia candelaris* (Meyen) Britton & Rose in Peru (Anderson, 2001).

Over-collection of cacti for subsequent sale has greatly affected some species. For example, the type locality (the place where the species was first found and recognized) of *Pelecyphora strobiliformis* near Miquihuana, México was virtually denuded of plants, which were dug up for sale in Europe (Sotomayor et al., 2004; Hernández and Gómez-Hinostrosa, 2005; Martorell and Peters, 2005). The great demand, primarily in USA markets, for saguaro ribs to make furniture is met by legally and illegally importing plants from México because this species is protected in the USA. Due to both gathering and livestock grazing, many candy barrel cacti continue to be destroyed *in situ* (Jiménez-Sierra and Eguiarte, 2010). Official inspections of private collections by German authorities led to the confiscation of 614 specimens of CITES Appendix I species, which were primarily Mexican cacti of apparently wild origin (Thiede, 2000).

Traditional cactus propagation and restrictions

Cacti species may be propagated as seeds, cuttings or by grafting (Santos-Díaz et al., 2010). Propagation using seeds allows for the maintenance of the genetic diversity of populations and species when the level of heterozygosity is high. Cactus seeds are typically very small with sizes varying between 0.5 and 5 mm, and 1 gram may comprise more than 1,000 seeds. However, in the wild, not all seeds develop into seedlings because predators consume a high number of the reproductive organs (Rojas-Aréchiga and Vázquez-Yanes, 2000). There is a number of cactus species in México and Latin America that produce sufficient viable seeds for efficient, rapid and cheap sexual propagation, which makes large-scale commercial propagation of them possible. These plants include *Mammillaria bombycina* Quehl, *Parodia leninhaussi* (K.Schumann) F.H.Brandt, *Ferocactus latispinus* Britton & Rose, *Myrthilocactus geometrizans* (Mart.) DC. and *Hylocereus undatus* (Haw.) Britton & Rose.

Several factors must be taken into account for seed germination to occur, such as the color, form, size, age, temperature, salinity, light, water potential, acidity, weight and origin of the seeds (Rojas-Aréchiga et al., 1997; Arias and Terrazas, 2004; Orozco-Segovia et al., 2007; Jiménez-Aguilar and Flores, 2010). For germination of cactus seeds, humidity and temperature are the most important factors. Seeds need little water to germinate, but once they are hydrated they should not be dried since they will die if this occurs. The optimum temperature for germination is 18-30 °C. After germination, soil temperature of 21-22 °C promotes healthy development of roots (Anderson, 2001).

Cacti are particularly sensitive to habitat disturbance and, in general, extreme temperatures do not favor germination, i.e., below 12 °C and above 36 °C (Nobel, 1988). Additionally, a number of cacti seeds exhibit dormancy; thus, physical (mechanical abrasion, cuttings, low temperature) and chemical scarification (immersion in concentrated HCl or H_2SO_4 acids or treatment with gibberellic acid) are employed to break seed dormancy (Rojas-Aréchiga et al., 2011). In the Opuntioideae subfamily in particular, seeds show low germination capacities and an extended period of time is required to complete germination primarily due to the structure of the ovule which consists of three integuments, the inner of which completely envelops the ovule (Mandujano et al., 2005).

Another method of propagating cacti is via vegetative propagation, which is usually easy, rapid and reliable (Santos-Díaz et al., 2010). In general, if the cactus plant has an elongated stem region that is actively growing, propagation via stem cuttings should be a successful alternative to seed propagation. It is recommended that any cut surfaces should be allowed to dry for a period of several days to several weeks until a callus forms over the cut surface. Rooting of cutting can then take place in an appropriate substrate, for example a mixture of 50 % peatmoss and 50 % sand, at a temperature of approximately 22 °C (López-Gómez et al., 2000; Bobich, 2005).

Cutting source and type, cicatrization time and stem-segment juvenility affect vegetative propagation. Genera members that can be propagated using stem cuttings include *Opuntias* (prickly pears, *Cylindropuntia*), columnar cacti (*Cereus* spp., *Trichocereus* spp., *Myrtillocactus*, *Hylocereus* and *Lophocereus*,) and a number of globular cacti (*Echinopsis* spp., *Mammillaria* spp.) (López-Gómez et al., 2000; Andrade and Martins, 2007; Cavalcante and Martins, 2008).

Cactus propagation by grafting is increasing, in particular in some Asian countries including Korea where huge numbers of cacti are produced annually using this procedure (Myeong et al., 2004). Nurseries, especially in Japan and Europe, have used grafting to bring unusual cacti growth forms to the market. Grafting is used for species that are difficult to grow well under cultivation or that cannot grow independently, such as certain chlorophyll-

free forms with white, yellow or red bodies, or other forms that show abnormal growth, to accelerate the growth of particularly slow-growing species, to save a damaged plant that is suffering from rot, to produce offsets to distribute plants rapidly or to save a particularly rare, delicate or special specimen. The plant rootstock source must grow well in cultivation and must be compatible with the plant being propagated: the scion. Compatibility between the rootstock and the scion is fundamental for grafting success. The growers make adequate cuts on the rootstock and scion and join both together to allow for the establishment of vascular connections and further cicatrization. Various types of grafts are used including flat grafts, where both the scion and rootstock are of similar diameters and cleft grafts, where a small scion is inserted onto a cleft made in the stock (Myeong et al., 2004; Bach, 2009).

Although vegetative propagation has some advantages over seed propagation of cactus species, there are also limitations; for example, vegetative reproduction is almost restricted to columnar cacti and the globular species are not amenable to being multiplied in this way. In addition, the number of plants that can be generated vegetatively is limited by the number of cladodes or "arms" in the adult plant which in the best case could be 100 propagules per plant, whereas some cacti species can produce several thousands of seeds such as *Ferocactus istrix* (DC.) G.E. Linds which is capable of producing approximately 300,000 seeds per plant per year. Vegetative reproduction could also lead to narrow genetic variation and, consequently, to a higher risk of massive attacks by pests or phytopathogenic agents.

In vitro propagation

In cases where over-collection in natural habitats has occurred, the starting material for cactus propagation is very limited or scarce. Micropropagation can be a useful tool for solving or overcoming these problems because sexual propagation is replaced by vegetative propagation cycles, which usually maintain the genetic fidelity of the starting material (cloning) and shorten the propagation period (usually from several months to a few weeks) (Malda et al., 1999b). In general, artificial growth media provide good conditions for either *in vitro* seed germination of almost all cactus species or for their micropropagation under aseptic conditions and controlled temperature, light and nutrition regimes (Shedbalkar and Adki, 2010). In some cases, full-strength MS (Murashige and Skoog, 1962) medium can inhibit or delay seed germination of cactus species (Lema-Rumińska and Kulus, 2012; Lema-Rumińska and Kulus, 2014).

In vitro culture establishment

Micropropagation of any plant species involves the selection of the explant donor followed by the elimination of surface microbial contamination of the tissues, inoculation in an appropriate culture media and incubation under controlled conditions to produce vegetatively a large number of plants. One of the primary issues in establishing *in vitro* cultures from outdoors or in greenhouse-maintained cactus plants is the elimination of bacterial and fungal contamination. Cactus plants growing outdoors or under greenhouse conditions may be used as explant sources to establish *in vitro* cultures; alternatively, *in vitro* plants raised from seeds have also been used as starting material for micropropagation of cacti (Lema-Rumińska and Kulus, 2012).

Clayton et al. (1990) established *in vitro* cultures from shoot-tip explants (1-2 cm in diameter) from primary or secondary stems of the rare or endangered species *Mammillaria wrightii* Engelm., *Pediocactus bradyi* L.D. Benson, *P. despainii* L.S. Welsh & Goodrich, *P. knowltonii* L.D. Benson, *P. paradinei* B.W. Benson, *P. winkleri* K.D. Heil and *Sclerocactus mesae-verdae* (Boissev. ex Hill & Salisb.) L.D. Benson mature plants and also from *in vitro*-germinated seedlings (0.5 cm in diameter) of *Escobaria missouriensis* (Sweet) D.R. Hunt, *E. robbinsorum* (W.H. Earle) D.R. Hunt, *Sclerocactus spinosior* (Engelm.) D. Woodruff & L.D. Benson and *Toumeya papyracantha* Britton & Rose. Explants were disinfected by immersion in 95 % ethanol for 1 min then in a 2 % commercial sodium hypochlorite (NaClO) solution for 7 min and were finally subjected to rinsing three times in sterile water. Estrada-Luna et al. (2008) used axillary buds from young cladodes (4-5 cm in length) excised from ornamental prickly pear *Opuntia lanigera* Salm-Dyck plants grown in a greenhouse to establish a micropropagation protocol. Spine-hairs were eliminated from the cladodes using scissors and they were treated with 70 % ethanol for 5 min and then immersed for 30 min in a commercial bleach solution (6 % NaClO) containing 0.1 % Tween-20. The cladodes were then washed with deionized sterile water (five times). Pairs of areoles were dissected from the cladodes and were used as explants.

Sriskandarajah and Serek (2004) also started *in vitro* cultures using phylloclade explants of *Schlumberguera* and *Rhipsalidopsis* from plants grown in the greenhouse. Due to the contamination issue frequently faced during the establishment of *in vitro* cultures of cacti species, many investigators have started from *in vitro* aseptic seedlings or other secondary explants axcissed from *in vitro* cultures. In general, seeds represent an excellent material because they can be subjected to high concentrations of commercial bleach solution to eliminate microbial contamination, and, thereby, aseptic seedlings may be obtained (Lema-Rumińska and Kulus, 2014).

In vitro seed germination is very often as high as 70-100 %. Aseptic seedlings have been used to establish *in vitro* cultures of *Escobaria missouriensis* (Sweet) D.R. Hunt, *E. robbinsorum* (W.H. Earle) D.R. Hunt, *Sclerocactus spinosior* (Engelm.) D. Woodruff & L.D. Benson, *Toumeya papyracantha* (Engelm.) N.P. Taylor (Clayton et al., 1990), *Cereus peruvianus* Engelm. & Bigelow (Oliveira et al., 1995), *Astrophytum asterias* (Zucc.) Lem. (Lema-Rumińska and Kulus, 2012), *Astrophytum myriostigma* Lem., *Cephalocereus senilis* (Haw.) Pfeiff, *Coryphantha clavata* (Scheidw.) Backeb, *C. durangensis* (Runge ex Schum.) Britton & Rose,

C. radians (De Candolle) Britton & Rose, *Echinocactus platyacanthus* Link & Otto, *Echinocereus dubius* (Engelm.), *E. pectinatus* (Scheidw.) Engelm., *Echinofossulocactus* sp. Lawr., *Ferocactus hamatacanthus* (Muehlenpf.) Britton & Rose, *F. histrix* (DC.) G.E. Linds., *F. latispinus* Britton & Rose, *F. pilosus* (Galeotti ex Salm-Dyck) Werdermann, *Mammillaria candida* (Scheidw.) Britton & Rose, *M. craigii* G.E. Linds., *M. formosa* (Scheidw.), *M. obscura* Hildm., *M. sphacelata* Mart., *M. uncinata* Zucc. ex Pfeiff., *Nyctocereus serpentinus* (Lag. & Rodr.) Britton & Rose, *Stenocactus coptonogonus* (Lem.) A. Berger (Pérez-Molphe-Balch et al., 1998), *Selenicereus megalanthus* (K. (Schum. ex Vaupel) Moran (Pelah et al., 2002; Quiala et al., 2009), *Pelecyphora aselliformis* Ehrenb. (Santos-Díaz et al., 2003), *Turbinicarpus laui* Glass & R.A. Foster, *T. lophophoroides* (Werdem.) Buxb. & Backeb., *T. pseudopectinatus* (Backeb.) Glass & R.A. Foster, *T. schmiedickeanus* subsp. *flaviflorus* (Gerhart Frank & A.B. Lau) Glass, *T. schmiedickeanus* subsp. *klinkerianus* (Backeb. & H. Jacobsen) N.P. Taylor, *T. schmiedickeanus* subsp. *schmiedickeanus* (Boed.) Rauh & Backeb., *T. subterraneus* (Backeb.) A.D. Zimmerman, *T. valdezianus* (H. Moeller) Glass & R.A. Foster (Dávila-Figueroa et al., 2005), *Notocactus magnificus* (F. Ritter) Krainz ex N.P.Taylor 1980 (Medeiros et al., 2006), and *Mammillaria mathildae* Kraehenb. & Krainz (García-Rubio and Malda-Barrera 2010), among others. However, *in vitro* seed germination may be limited because some cactus species exhibit seed dormancy (Lema-Rumińska and Kulus, 2014); for example, Cardarelli et al. (2010) used 1.4 μM GA_3 to overcome seed dormancy in the endangered species *Obregonia denegrii* Fric, and they achieved higher germination (85 %) after 7 days of culture on MS medium than from control seeds that were soaked in distilled water (22 %).

Growth regulators for cacti tissue culture

Responses of cells, tissues and organs cultured *in vitro* depend upon both endogenous levels of plant hormones and the exogenous growth regulators added to the growth medium. When an explant is inoculated into a growth medium, the responses to exogenous growth regulators depend to a great extent on the physiological status of the donor plant and the origin and the cellular types in the explant itself as well as many other factors. Each tissue cultured *in vitro* exhibits a specific response in the presence of a particular growth regulator or combinations of different types and levels of growth regulators.

Plant growth regulators are used to induce direct or indirect organogenesis and embryogenesis. The growth regulators most commonly used to induce direct organogenesis (*de novo* direct organ generation, i.e. shoots or roots from the cultured explant without an intermediate callus step formation) in cactus species are cytokinins, and more specifically, 6-benzyladenine (BA), but other cytokinins, such as kinetin (Kin), 2-isopentenyl adenine (2iP) and zeatin can eventually induce callus formation in some cacti species depending on the concentration and combination with auxins (Lema-Rumińska and Kulus, 2014). Growth media supplemented with thidiazuron (TDZ) have been utilized only very occasionally, but this growth regulator is becoming more and more popular. The auxin incorporated most frequently into growth media to induce organogenesis is 1-naphthaleneacetic acid (NAA) at 2.7 μM (Pérez-Molphe-Balch et al., 1998; Mata Rosas et al., 2001; Moebius-Goldammer et al., 2003) followed by indole-3-acetic acid (IAA) at 0, 5.7, 11.4 and 22.8 μM, and indole-3-butyric acid (IBA). In general, 2,4-dichlorophenoxyacetic acid (2,4-D) is useful when the aim is to promote callus formation and/or somatic embryogenesis in different explants and plant species.

When seeds of cactus species exhibit dormancy or when the regenerated shoots require an elongation step, the growth medium is typically supplemented with gibberellic acid (GA_3). Abscisic acid (ABA) has been reported to have beneficial effects on the maturation process of somatic embryos or occasionally, to prevent their germination. Concentrations of growth regulators in the growth medium used for cactus species range from 0, as in the case of *in vitro* culture of *Mammillaria gracilis* Pfeiff. (Balen et al., 2004), to low, intermediate or high levels of cytokinins, such as BA (4.4, 6.7, 8.8, 13.3 and 44.4 μM), employed alone in several species of *Mammillaria* (*Mammillaria longimamma*, *Mammillaria stylothele*), *Turbinicarpus* and *Escobaria vivipara*, or in combinations with an auxin like NAA (2.7-10.7 μM) or IAA (2.9-5.7 μM) with *Notocactus* (Mata Rosas et al., 2001; Dávila-Figueroa et al., 2005; Medeiros et al., 2006; Quiala et al., 2009). Growth media have been supplemented with Kin at 0, 4.6, 13.9, 22.3, 27.9 and 46.5 μM in the absence or presence of an auxin (IAA or NAA), and zeatin has been added at 4.6 and 22.3 μM for *in vitro* multiplication of species of the genera *Pediocactus*, *Sclerocactus* and *Toumeya* (Clayton et al., 1990).

In vitro multiplication step

By areole activation (meristematic tissues)

A number of cactus species have been found to regenerate entire plants by activating meristematic cells in the areoles via cytokinins alone or in combination with low levels of auxins (Lema-Rumińska and Kulus, 2014). An areole is a highly specialized axillary bud that contains meristematic tissues. New shoots and lateral branches in cactus originate from these structures. Areoles are unique structures in these plants that also produce spines, trichomes and flowers. When cactus tissues are cultured *in vitro* in the presence of cytokinins, occasionally in combination with low concentrations of auxins, areoles may sprout and form one or multiple new shoots (Figure 1). After being separated from the original tissue, these shoots may be rooted and grown into new plants, which is a simple and fast system for propagating cactus species *in vitro*. This multiplication pathway is also called areole activation and is the method most widely used for *in vitro* mass propagation of cacti. Species that have been propagated using areole activation with cytokinin/auxin treatments are shown in Table 2.

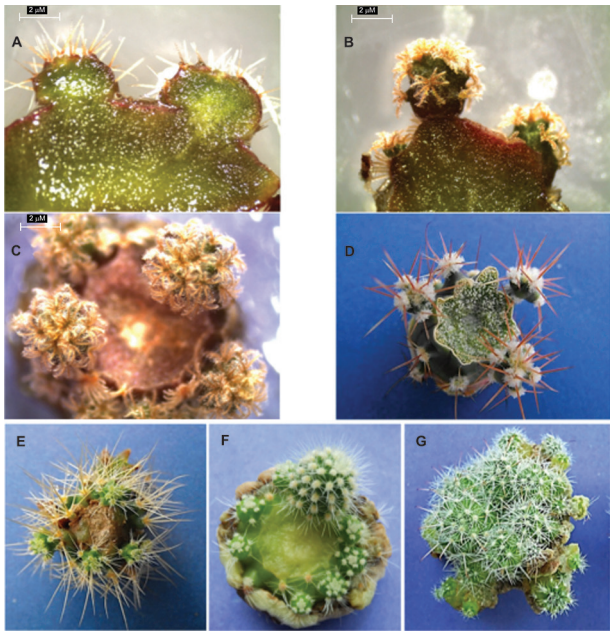

Figure 1 – *In vitro* propagation of several cactus species via areole activation. A slice of a *Frailea castanea* explant cultured with 2.2 µM 6-benzyladenine (BA) showing two sprouting areoles (A); areoles of *Turbinicarpus pseudopectinatus* sprouting on medium containing 4.4 µM BA (B and C); areole activation in *Ferocactus emory* ssp. *rectispinus* with 9.8 µM 2iP (D), *Ferocactus histrix* with 8.8 µM BA (E), *Echinocereus ferreirianus* ssp. *lindsay* with 4.4 µM BA (F), and *Mammillaria jaliscana* with 2.2 µM BA (G).

Any portion of plant tissue containing areoles may be used as an explant for the multiplication step. The method used to prepare the explant determines the number of areoles and thus the number of shoots obtained. The efficiency of this multiplication system, measured as the number of new shoots generated per explant usually varies depending on the species, the explants and treatments used. As shown by the reports cited in Table 2, the lower efficiencies range from two and three shoots per explant whith the highest being close to 30 shoots produced per explant in a single culture cycle. However, if the explants that generate the shoots are maintained on a medium that contains cytokinins in culture vessels, areoles in these new shoots can in turn sprout producing secondary proliferation. Thus, it is possible to obtain mean efficiencies in excess of 120 shoots per explant (Pérez-Molphe-Balch and Dávila-Figueroa, 2002).

An additional advantage of this technique is that somaclonal variation and genetic alterations in the regenerated plants are generally absent or are found with low frequency compared with other regeneration systems due to the use of meristematic explants and low concentrations of plant growth regulators. In general, no callus is formed and only pre-existing meristems are activated (Vyscot and Jára, 1984). Clayton et al. (1990) reported axillary shoot regeneration from shoot-tip explants of the rare or endangered the cactus species *Escobaria missouriensis* (Sweet) D.R. Hunt, *E. robbinsiorum* (W.H. Earle) D.R. Hunt, *Sclerocactus spinosior* (Engelm.) D. EWoodruff & L.D. Benson, *Toumeya papyracantha* (Engelm.) Britton & Rose, *Mammillaria wrightii* Engelm., *Pediocactus bradyi* L.D. Benson, *P. despainii* S.L. Welsh & Goodrich, *P. knowltonii* L.D. Benson, *P. paradinei* B.W. Benson, *P. winkleri* K.D. Heil and *Sclerocactus mesaeverdae* (Boissev. & C. Davidson) L.D. Benson cultured on MS, B5, SH and L2 basal media (Gamborg et al., 1968; Schenk and Hildebrandt, 1972; Phillips and Collins, 1979) or MS basal medium supplemented with different cytokinins (adenine sulfate, BA, 2iP, Kin and zeatin) and auxins [IAA, IBA, NAA and 4-amino-3,5,6-trichloropicolinic acid (Picloram)]. They observed that the basal L2 medium was superior, but still MS with low or moderate-to-high cytokinin concentrations (22.8 to 49.2 µM) is the most popular and induced the greatest shoot formation. For all species tested, the combination 45.6 µM zeatin + 1.1 µM NAA was optimal for axillary shoot production.

Machado and Prioli (1996) reported the production of axillary shoots from *Cereus peruvianus* areoles starting from lateral explants cultured on MS medium containing 4.44 µM BA and 5.71 µM IAA or 5.37 µM NAA. Pérez-Molphe-Balch et al. (1998) reported that *in vitro* cultures of *Astrophytum myriostigma* Lem., *Cephalocereus senilis* (Haw.) Pfeiff., *Coryphantha clavata* (Scheidw.) Backeb., *C. durangensis* (Runge ex K. Schum.) Britton & Rose, *C. radians* (DC.) Britton & Rose, *Echinocactus platyacanthus* Link & Otto, *Echinocereus dubius* (Engelm.) Engelm. ex Rümpler, *E. pectinatus* (Scheidw.) Engelm., *Echinofossulocactus* sp. Lawr., *Ferocactus hamatacanthus* (Muehlenpf.) Britton & Rose, *F. histrix* (DC.) G.E. Linds., *F. latispinus* (Haw.) Britton & Rose, *F. pilosus* (Galeotti) Werderm., *Mammillaria candida* Scheidw., *M. craigii* G.E. Linds., *M. formosa* Scheidw., *M. obscura* Hildm., *M. sphacelata* Mart., *M. uncinata* Zucc. ex Pfeiff., *Nyctocereus serpentinus* (Lag. & Rodr.) Britton & Rose, *Stenocactus coptonogonus* (Lem.) A. Berger exhibited multiple shoot formations from areoles on MS culture medium with 4.4 or 8.9 µM BA alone or in combination with 0.54 or 5.4 µM NAA.

In another study, Pérez-Molphe-Balch and Dávila-Figueroa (2002) established *in vitro* cultures of the Mexican endangered cacti *Pelecyphora aselliformis* Ehrenb. and *Pelecyphora strobiliformis* (Werderm.) Kreuz., and they observed shoot formations from hormone-activated areoles after 60 days of incubation on MS medium in the presence of different cytokinins. BA was more effective than either 2iP or thidiazuron for activating axillary buds. *P. aselliformis* and *P. strobiliformis* produced 13.7 and 12.4 shoots per apical explant, respectively, in the presence of 8.8 µM BA. Dávila-Figueroa et al. (2005) achieved multiple shoot formations in eight species of *Turbinicarpus* cultured on MS medium containing cytokinins with efficiencies ranging from 7.8 shoots per explant for *T. valdezianus* (H. Moeller) Glass & R.A. Foster to 19.7 for *T. pseudopectinatus* (Backeb.) Glass & R.A. Foster using BA or 2iP.

Table 2 – Reports of *in vitro* propagation of cacti via areole activation.

Species	Treatments that gave the best results	Shoots per explant	Reference
Astrophytum myriostigma	6-benzyladenine (BA) (22.2 µM) + 1-naphthaleneacetic acid (NAA) (5.4 µM)	2.0	Vyscot and Jára (1984)
Mammillaria carmenae	BA (8.9 µM) + NAA (5.4 µM)	2.8	
M. prolifera	BA (2.2 µM) + NAA (5.4 µM)	2.1	
Trichocereus spachianus	BA (22.2 µM) + NAA (5.4 µM)	3.0	
Opuntia amyclaea	BA (10 µM)	15.0	Escobar et al. (1986)
Mammillaria haageana subsp. *san-angelensis*	BA (4.4 µM)	9.0	Martínez-Vázquez and Rubluo (1989)
E. robbinsorum	Zeatin (45.6 µM) + Picloram (0.8 µM)	4.3	Clayton et al. (1990)
Mammillaria wrightii	Zeatin (22.8 µM) + NAA (1.1 µM)	3.8	
Pediocactus bradyi	Zeatin (22.8 µM)	7.1	
P. despaini	Zeatin (22.8 µM)	6.9	
P. knowltonii	Zeatin (22.8 µM)	3.9	
P. winkleri	Zeatin (45.6 µM) + Picloram (0.8 µM)	9.8	
Sclerocactus mesae-verdae	Kinetin (Kin) (46.4 µM) + Picloram (0.8 µM)	4.3	
S. spinosior	Zeatin (22.8 µM)	2.3	
Toumeya papyracantha	Zeatin (22.8 µM)	3.6	
Mediocactus coccineus	BA (4.4 µM) + NAA (0.27 µM)	7.8	Infante (1992)
Cereus peruvianus	BA (4.4 µM) + indole-3-acetic acid (IAA) (5.71 µM)	1.0	Machado and Prioli (1996)
Astrophytum myriostigma	BA (4.4 µM) + NAA (0.54 µM)	9.2	Pérez-Molphe-Balch et al. (1998)
Cephalocereus senilis	BA (4.4 µM) + NAA (0.54 µM)	2.8	
Coryphantha clavata	BA (4.4 µM)	4.7	
C. durangensis	BA (4.4 µM) + NAA (0.54 µM)	4.4	
C. radians	BA (4.4 µM)	4.1	
Echinocactus platyacanthus	BA (4.4 µM)	9.0	
Echinocereus dubius	BA (4.4 µM) + NAA (0.54 µM)	4.9	
E. pectinatus	BA (4.4 µM) + NAA (0.54 µM)	3.9	
Echinofossulocactus sp.	BA (4.4 µM)	12.0	
Ferocactus hamatacanthus	BA (4.4 µM) + NAA (5.4 µM)	5.8	
F. histrix	BA (4.4 µM) + NAA (0.54 µM)	5.6	
F. latispinus	BA (4.4 µM) + NAA (5.4 µM)	5.3	
F. pilosus	BA (4.4 µM) + NAA (5.4 µM)	5.1	
Mammillaria candida	BA (4.4 µM)	13.2	
M. craigii	BA (4.4 µM)	4.6	
M. formosa	BA (4.4 µM) + NAA (5.4 µM)	4.4	
M. obscura	BA (4.4 µM) + NAA (5.4 µM)	4.8	
M. sphacelata	BA (4.4 µM) + NAA (0.54 µM)	17.5	
M. uncinata	BA (4.4 µM)	5.2	
Nyctocereus serpentinus	BA (8.9 µM)	2.1	
Stenocactus coptonogonus	BA (4.4 µM)	16.7	
Pelecyphora aselliformis	BA (8.9 µM)	13.7	Pérez-Molphe-Balch and Dávila-Figueroa (2002)
P. strobiliformis	BA (8.9 µM)	12.4	
Carnegiea gigantea	BA (8.9 µM)	5.3	
Pachycereus pringlei	BA (4.4 µM)	3.8	
Stenocereus thurberi	BA (4.4 µM)	4.3	
Acharagma aguirreana	2-isopentenyladenine (2iP) (9.8 µM)	5.8	Castro-Gallo et al. (2002)
Astrophytum ornatum	BA (21.5	11.0	
Coryphantha elephantidens	BA (8.9 µM)µM) + NAA (0.54 µM)	2.9	
Ferocactus flavovirens	2iP (29.5 µM)	6.3	
Mammillaria bocasana	BA (8.9 µM)	4.8	
M. oteroi	2iP (19.6 µM)	4.8	
Pachycereus schottii	BA (13.3 µM)	4.3	
Pilosocereus chrysacanthus	BA (8.9 µM)	9.6	
Stenocereus stellatus	BA (4.4 µM)	7.0	
Thelocactus hexaedophorus	2iP (29.5 µM)	13.6	

Continue.

Table 2 - Continuation.

Hylocereus undatus	Thidiazuron (TDZ) (0.5 µM) + NAA (0.5 µM)	8.7	Mohamed-Yasseen (2002)
Escobaria minima	TDZ (2.27 µM)	3.6	Giusti et al. (2002)
Mammillaria pectinifera	TDZ (2.27 µM)	3.5	
Pelecyphora aselliformis	Kin (23.2 µM) + NAA (0.05 µM)	10.2	
Opuntia ellisiana	BA (10 µM) + indole-3-butyric acid (IBA) (10 µM)	1.0	Juárez and Passera (2002)
Astrophytum asterias	BA (2.2 µM)	2.3	Lizalde et al. (2003)
Echinocactus grusonnii	BA (8.9 µM)	4.1	
Coryphantha werdermannii	BA (8.9 µM)	3.1	
Echinocereus adustus	BA (4.4 µM)	6.1	
E. delaetii	BA (4.4 µM)	4.1	
E. ferreirianus	BA (4.4 µM)	6.4	
Epithelantha micromeris	BA (2.2 µM)	6.5	
Ferocactus cylindraceus	BA (11.1 µM)	4.4	
Morangaya pensilis	BA (2.2 µM)	3.9	
Opuntia ficus-indica	BA (2.2 µM)	13.8	García-Saucedo et al. (2005)
Turbinicarpus laui	BA (4.4 µM)	16.8	Dávila-Figueroa et al. (2005)
T. lophophoroides	BA (8.9 µM)	8.8	
T. pseudopectinatus	BA (3.3 µM)	19.7	
T. schmiedickeanus subsp. *flaviflorus*	2iP (19.7 µM)	13.7	
T. schmiedickeanus subsp. *klinkerianus*	2iP (24.6 µM)	13.6	
T. schmiedickeanus subsp. *schmiedickeanus*	BA (8.9 µM)	10.0	
T. subterraneus	BA (4.4 µM)	12.8	
T. valdezianus	BA (2.2 µM)	7.8	
Browningia candelaris	Meta-Topolin (2.1 µM)	20.4	Sánchez-Morán and Pérez-Molphe-Balch (2007)
Echinocereus knippelianus	BA (8.9 µM)	8.9	
Echinocereus schmollii	BA (8.9 µM)	13.5	
Escontria chiotilla	BA (8.9 µM)	7.6	
Mammillaria carmenae	BA (8.9 µM)	7.7	
M. carmenae f. rubrisprina	BA (4.4 µM)	6.0	
M. herrerae	BA (2.2 µM)	8.1	
M. theresae	2iP (24.6 µM)	8.2	
Melocactus curvispinus	2iP (24.6 µM)	11.8	
Polaskia chichipe	BA (4.4 µM)	11.5	
Hylocereus costaricensis	BA (30 µM)	1.0	Viñas et al. (2012)
Turbinicarpus bonatzii	2iP (9.8 µM)	11.3	De la Rosa Carrillo et al. (2012)
T. hoferi	BA (4.4 µM)	4.0	
T. jauernigii	BA (5.5 µM)	4.4	
T. pseudomacrochele subsp. *lausseri*	BA (3.3 µM)	26.3	
T. pseudomacrochele subsp. *pseudomacrochele*	BA (5.5 µM)	12.2	
T. rioverdensis	BA (4.4 µM)	4.3	
T. roseiflorus			
T. schmiedickeanus subsp. *dickisoniae*	2iP (4.9 µM)	11.0	
T. schmiedickeanus subsp. *gracilis*	BA (6.7 µM)	9.4	
T. schmiedickeanus subsp. *macrochele*	BA (2.2 µM)	10.1	
T. schmiedickeanus subsp. *schwarzii*	2iP (9.8 µM)	11.9	
T. swobodae	BA (2.2 µM)	5.5	
T. valdezianus subsp. *albiflorus*	2iP (4.9 µM)	11.1	
T. ysabelae	BA (2.2 µM)	10.1	

Retes-Pruneda et al. (2007) developed micropropagation systems by areole activation for *Echinocereus knippelianus* Liebm., *E. schmollii* (Weing.) N.P. Taylor, *Mammillaria carmenae* Castañeda (normal and rubrispina forms), *M. herrerae* Werderm., *M. theresae* Cutak, *Melocactus curvispinus* Pfeiff., *Escontria chiotilla* (F.A.C. Weber) Rose

and *Polaskia chichipe* (Rol.-Goss.) Backeb. using MS medium containing BA (0, 2.22, 4.44 and 8.88 μM) or 2iP (0, 4.92, 14.8 and 24.6 and 24.6 μM). The greatest responses were obtained with the highest cytokinin levels. Estrada-Luna et al. (2008) developed a micropropagation protocol for the ornamental prickly pear cactus *Opuntia lanigera* Parm. ex Pfeiff. The explants tested were placed in vertical or horizontal positions on MS medium with different cytokinins (0, 5.55, 11.1, 22.2 and 31.1 μM BA; 0, 6.15, 12.3, 24.6 and 34.4 μM 2iP; and 0, 5.7, 11.4, 22.8 and 31.9 μM Kin) for the activation of areoles to produce axillary shoots. It was observed that vertical orientation was optimal (approximately 5 shoots per explant) compared with horizontal (3.7 shoots per explant) and that the highest shoot regeneration was achieved with BA (8 shoots per explant) compared with 2iP and Kin (2 shoots per explant).

Cardarelli et al. (2010) observed multiple shoot formations from areoles of the endangered cactus *Obregonia denegrii* Fric on MS medium containing 4.4 μM BA and 10.7 μM NAA together with *N*-(2-chloro-4-pyridyl)-*N*-phenylurea (4-CPPU), a type of cytokinin. Ruvalcaba-Ruiz et al. (2010) tested half-strength MS medium supplemented with BA (0, 4.44, 8.88 and 13.3 μM) and NAA (0, 2.69 and 5.37 μM) to micropropagate the Mexican endemic and endangered ornamental cactus *Coryphanta retusa* (Pfeiff.) Britton & Rose. The highest shoot regeneration was recorded in the presence of 8.88 μM BA after 60 days of incubation.

Rosa-Carrillo et al. (2012) reported micropropagation following activation of areoles in tissue cultures of 14 species and subspecies of *Turbinicarpus* with efficiencies of 4.0 shoots per explant for *T. hoferi* Lüthy & A.B. Lau in the presence of 4.4 μM BA and of 26.5 for *T. pseudomacrochele* subsp. *lausseri* (Diers & G. Frank) Glass with 3.3 μM BA after 60 ± 5 days of incubation. *T. hoferi*, *T. jauernigii* G. Frank, *T. pseudomacrochele* subsp. *lausseri*, *T. pseudomacrochele* subsp. *pseudomacrochele* (Backeb.) Rauh & Backeb., *T. schmiedickeanus* subsp. *gracilis* (Glass & R.A. Foster) Glass, *T. swobodae* Diers and *T. ysabelae* (Schlange) John & Riha responded better when BA was present in the growth medium, whereas *T. bonatzii* G. Frank, *T. schmiedickeanus* subsp. *dickisoniae* (Glass & R.A. Foster) N.P. Taylor, *T. schmiedickeanus* subsp. *schwarzii* (Shurly) N.P. Taylor and *T. valdezianus* subsp. *albiflorus* (Møller) Glass & R.A. Foster showed greater shoot formation with 2iP, and *T. rioverdensis* G. Frank, *T. roseiflorus* Backeb. and *T. schmiedickeanus* subsp. *macrochele* (Werderm.) N.P. Taylor were not affected differently by these two cytokinins.

The most frequently studied factor for *in vitro* regeneration of cactus species from areoles are growth regulators. In most reports, the Murashige and Skoog basal medium has been the only one tested or used. No studies on the effect of physical factors such as light and temperature of incubation have been reported.

Via organogenesis

In vitro organogenesis is defined as the *de novo* induction and generation of organs from cells, tissues or organs cultured under aseptic and controlled conditions. Organogenesis may occur directly from cells, tissues or organs in culture or indirectly from the intermediate step of callus formation (de-differentiation) and then new-organ formation. As a general rule, two steps are involved in *in vitro* plant regeneration via organogenesis: shoot formation and root induction.

In general, *in vitro* indirect organogenesis occurs when high auxin or cytokinin (especially BA) levels are present in the growth medium during the initial step that promotes callus formation. Adventitious buds are regenerated after transfer to a medium containing a high cytokinin/auxin ratio. For example, Oliveira et al. (1995) regenerated plants from friable callus tissue formed in seedling explants cultured on MS medium containing combinations of 18.1 μM 2,4-D and 18.6 or 27.9 μM Kin. Wakhlu and Bhau (2000) induced callus from the pith tissue of *Coryphantha elephantidens* (Lem.) Lem. inoculated onto MS growth medium with 9.1 μM 2,4-D and 2.3 μM Kin, and subsequently regenerated shoots after four weeks of incubation on growth medium supplemented with 6.9 μM kinetin and 2.3 μM 2,4-D. The callus tissue retained its regenerative capability over a lengthy period (18 subcultures), and the regenerated plants were morphologically similar to the explant donor plants. Papafotiou et al. (2001) reported indirect shoot regeneration from calli formed in tubercle explants of *Mammillaria elongata* DC. cultured on MS medium with 1.1 μM NAA and 22.2 μM BA.

Giusti et al. (2002) investigated the responses of explants from *Escobaria minima* (Baird) D.R. Hunt, *Mammillaria pectinifera* F.A.C. Weber and *Pelecyphora aselliformis* Ehrenb. cultured on MS medium containing 2.3 μM thidiazuron; they observed callus formation and then shoot regeneration (3.0, 3.5 and 10.2 shoots per explant of each species, respectively). Interestingly, Poljuha et al. (2003) reported that *in vitro*-propagated shoots of *Mammillaria gracillis* Pfeiff. developed callus spontaneously, which in turn regenerated normal and hyperhydric shoots in the absence of exogenous growth regulators. More recently, García-Rubio and Malda-Barrera (2010) described a micropropagation protocol for the endangered endemic cactus *Mammillaria mathildae* Kraehenb. & Krainz using basal explants that produced callus on MS medium containing combinations of BA (0, 22.2 and 44.4 μM) and IAA (0, 1.4, 2.9 and 5.7 μM), from which shoots were regenerated.

Organogenesis in 13 cacti species

We have previously mentioned the establishment of micropropagation protocols for 50 cacti species in our laboratories, and as an example, protocols for the *in vitro* propagation of 13 species are described here. Explants (stem segments bearing at least three areoles) from *Cleistocactus straussi* (Heese) Backeb., *Coryphantha georgii* Boed., *Echinocactus platyacanthus* Link & Otto, *Ferocactus hamatacanthus* (Muehlenpf.) Britton & Rose, *Mammillaria haageana* subsp. *san-angelensis* (Sánchez-Mej.)

D.R. Hunt, *Mammillaria petterssonii* Hildm., *Melocactus matanzanus* León and *Opuntia erinacea* Engelm. & J.M. Bigelow showed direct organogenesis, whereas *Astrophytum myriostigma* Lem., *Cephalocereus senilis* (Haw.) Pfeiff., *Echinocactus grusonii* Hildm., *Echinocereus pectinatus* (Scheidw.) Engelm. and *E. schmolli* (Weing.) N.P. Taylor exhibited indirect organogenesis.

In vitro cultures of *Cephalocereus senilis* and *Mammillaria petterssonii* were established previously at Aguascalientes, Ags., Mexico. They were micropropagated by subculturing cross sections of the *in vitro* generated shoots on MS medium with 4.4 μM BA + 0.054 μM NAA (Pérez-Molphe-Balch et al., 1998) to increase the plant number to a total of approximately 200 shoots (1 g fresh weight each) to establish factorial growth-regulator treatment experiments. *In vitro* cultures of *Astrophytum myriostigma*, *Echinocactus platyacanthus* and *Melocactus matanzanus* were initiated from aseptic seedlings obtained by germinating disinfected seeds treated with 0.58 mM GA_3 solution for 2 h. The treated seeds were surface sterilized using 20 % (v/v) commercial bleach for 20 min, rinsed with sterile distilled water and finally inoculated into basal MS medium. After germination, the seedlings were cultured on MS medium containing 9 μM BA + 2.9 μM IAA as a pre-treatment to promote growth and multiplication. When seedlings reached approximately 1 g fresh weight and the availability of explants was approximately 200, factorial experiments were established to test different types, combinations and concentrations of growth regulators for micropropagation.

In vitro cultures of *Cleistocactus straussi*, *Coryphantha georgii*, *Echinocactus grusonii*, *Echinocactus schmollii*, *Echinocereus pectinatus*, *Ferocactus hamatacanthus*, *Mammillaria haageana* sbsp. *san-angelensis* and *Opuntia erinacea* were initiated using plants from commercial nurseries as explant sources. These plants were washed with soap and tap water, rinsed and treated with Benomyl solution (1 g L^{-1}) for 2 h. They were then placed in pots of sterile soil and maintained under greenhouse conditions for 6 months to reduce the number of contaminating microorganisms, which very often make the establishment of *in vitro* cultures difficult. Following this treatment, fragments of tissue (2-5 g fresh weight), including meristems, were dissected from the top of each plant. These tissue sections were washed with sterile distilled water and maintained for 5 h in a laminar air flow cabinet to allow for elimination of excess humidity (to approximately 20 % fresh weight). The tissue sections were surface sterilized by treating with 70 % ethanol (v/v) for 60 s and then with 20 % (v/v) of a commercial disinfectant solution containing 0.048 % silver colloid (Roland de México, S.A. de C.V.) for 20 min. Finally, the tissues were treated with a 20 % (v/v) commercial bleach solution containing 0.1 % Tween 20 for 20 min and rinsed 5 times with sterile distilled water. Damaged tissue areas were eliminated, and each healthy tissue section was cut into 4 explants (approximately 1 g fresh weight each) bearing meristematic tissue, which were subsequently cultured on MS medium containing 13.9 μM Kin, 2.9 μM IAA, 3 % sucrose, adjusted to pH 5.7-5.8, and gelled using 8 g L^{-1} agar. Incubation of the explants proceeded under a photoperiod (16 h light/8 h dark; daylight fluorescent lamps; 15 μmol m^{-2} s^{-1}) at 25 ± 2 °C. Depending on the cactus species, the time required to obtain 150-200 explants prior to the establishment of factorial experiments was approximately 6-12 months.

Two experimental groups were prepared using two combinations of growth regulators. For the first group, 15 treatments were tested using NAA (0, 0.05 and 0.53 μM) combined with BA (0, 4.4, 13.3, 26.7 and 44.4 μM) for the species *Echynocactus grusonii*, *Echynocactus platyacanthus*, *Mammillaria petterssonii*, *M. haageana* subsp. *san-angelensis* and *Opuntia erinacea*. Each treatment comprised 10 replicates (one explant per 120 mL glass bottle containing 20 mL culture medium) and one additional subculture on the same fresh medium for 30 days in both cases. The number of shoots per explant was recorded after 60 days in culture, and the results were analyzed statistically using ANOVA followed by the the Mean Separation Test using a Minimum Significant Difference where $\alpha = 0.05$ and the Statgraphics program version 2.5.

The second group consisted of 20 treatments using IAA (0, 5.7, 11.4 and 22.8 μM) combined with Kin (0, 4.6, 13.9, 27.9 and 46.5 μM) for the following species: *Astrophytum myriostigma*, *Cephalocereus senilis*, *Cleistocactus straussi*, *Coryphantha georgii*, *Echinocactus grusonii*, *Echinocactus platyacanthus*, *Echinocereus pectinatus*, *Echinocereus schmollii*, *Ferocactus amatacanthus*, *Melocactus matanzanus*. The same number of replicates, experimental conditions, response evaluation time and statistical analysis as used for group one were also used for this second group.

Shoots were cultured on half-strength MS medium without growth regulators to induce roots. When rooted plants reached 1-2 cm in length, they were collected, washed with tap water to eliminate agar residues and left to dehydrate at room temperature for 10 days. The plants were then treated with a 2 g L^{-1} Captan + 1 g L^{-1} Benomyl suspension for 2 h, and a rooting powder (Rootone) was applied at the base to stimulate further root formation. These treated plants were placed in pots (7.5 cm in diameter) in a sterilized substrate (mixture of 50 % perlite and 50 % peat moss), and they were maintained under greenhouse conditions during spring and summer from March to the end of August.

The factorial experiments using NAA/BA showed clearly that cactus species exhibited different *in vitro* shoot regeneration capacities; for example, *Mammillaria petterssonii*, *M. haageana* sbsp. *san-angelensis* and *Opuntia erinacea* showed a maximum response (9.6, 17 and 12.6 shoots per explant, respectively) on culture media supplemented with 0.53 μM NAA + 26.7 μM BA, 17 μM BA and 4.4 μM BA, respectively, in experiments with ten replicates over two experiments (Table 3). *Echynocactus grusonii* and *Echynocactus platyacanthus* showed lower shoot formation than the three species above with maxi-

mum values of only 2.7 and 3.5 shoots per explant on culture media containing 0.53 µM NAA + 26.7 µM BA and 0.53 µM NAA + 4.4 µM BA, respectively (Table 3).

Because the *in vitro* responses of *E. grusonii* and *E. platyacanthus* explants were not satisfactory when NAA/BA combinations were used, 20 treatments of IAA/Kin were assayed, and 8 additional cactus species were also tested (Tables 4 and 5). Shoot formation in *E. grusonii* explants increased from the former value of 2.7 with the NAA/BA combinations (Table 3) to 7.5 shoots per explant on MS culture medium supplemented with 22.8 µM IAA + 27.9 µM Kin (Table 4). Shoot formation from *E. platyacanthus* also increased from 3.5 in NAA/BA (Table 3) to 5.6 shoots per explant on MS medium containing 2.8 µM IAA + 13.9 µM Kin (Table 5). Growth medium supplemented with 11.4 µM IAA + 46.5 µM Kin was effective for maximum shoot induction in *Astrophytum myriostigma*, *Cephalocereus senilis*, *Cleistocactus straussi* and *Coryphanta georgii* (Figure 2), which exhibited 7.8, 6.7, 10.7 and 7.2 shoots per explants, respectively (Table 4).

Table 3 – *In vitro* shoot regeneration in explants from five cactus species cultured on Murashige and Skoog (MS) medium containing combinations of 1-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) after 60 days of incubation.

NAA/BA (µM)	*Echynocactus grusonii*	*Echynocactus platyacanthus*	*Mammillaria petterssonii*	*Mammillaria haageana* subsp. *san-angelensis*	*Opuntia erinacea*
0/0	0.7 bcd	0.8 bc	2.1 de	1.5 ef	1.0 d
0/4.4	1.1 bcd	1.9 b	1.2 de	2.3 def	12.6 a
0/13.3	2.0 ab	0.5 c	5.7 bc	7.4 cd	9.5 abc
0/26.7	1.7 abc	0.8 bc	6.3 abc	17.0 a	8.4 bc
0/44.4	0.9 bcd	0.7 bc	8.7 ab	4.8 cdef	7.1 c
0.05/0	0.3 cd	1.0 bc	0.2 e	1.0 f	0.6 d
0.05/4.4	0.9 bcd	0.9 bc	1.6 de	1.3 ef	12.5 a
0.05/13.3	0.3 cd	1.8 bc	5.8 bc	6.1 cdef	11.8 ab
0.05/26.7	1.4 abcd	1.3 bc	8.9 ab	9.7 bc	8.5 bc
0.05/44.4	1.0 bcd	0.8 bc	7.2 abc	6.5 cde	6.5 c
0.53/0	0.3 cd	1.2 bc	1.1 de	1.0 f	0.5 d
0.53/4.4	0.3 cd	3.5 a	0.4 e	4.6 cdef	12.5 a
0.53/13.3	1.4 abcd	0.9 bc	4.4 cd	7.4 cd	11.8 ab
0.53/26.7	2.7 a	1.1 bc	9.6 a	15.1 a	6.3 c
0.53/44.4	1.0 bcd	1.4 bc	8.4 ab	12.7 ab	7.7 c

Values with the same letter are not different at 5 %, determined by the LSD test (Fisher test).

Table 4 – *In vitro* shoot regeneration in explants from *Astrophytum myriostigma*, *Cephalocereus senilis*, *Cleistocactus straussi*, *Coryphantha georgii and Echinocactus grusonii* cultured on Murashige and Skoog (MS) medium containing combinations of indole-3-acetic acid (IAA)/kinetin (Kin) after 60 days of incubation.

IAA/Kin (µM)	*Astrophytum myriostigma*	*Cephalocereus senillis*	*Cleistocactus straussi*	*Coryphantha georgii*	*Echinocactus grusonii*
0/0	1.0 b	1.1 d	0.9 f	1.0 c	1.5 e
0/4.6	1.4 b	1.2 d	1.6 f	2.8 bc	3.7 bcde
0/13.9	5.2 ab	1.7 cd	2.8 ef	5.0 abc	4.7 abcd
0/27.9	5.2 ab	2.l cd	5.6 cd	4.2 abc	6.3 ab
0/46.5	3.0 ab	2.2 cd	6.8 bc	3.4 abc	5.8 abc
5.7/0	1.1 b	1.6 cd	1.5 f	2.0 c	1.8 de
5.7/4.6	1.2 b	1.3 d	2.3 f	1.2 c	3.0 cde
5.7/13.9	2.8 ab	2.0 cd	6.3 bc	4.6 abc	5.0 abc
5.7/27.9	2.6 ab	6.2 ab	6.9 bc	6.4 ab	6.3 ab
5.7/46.5	4.6 ab	2.4 cd	7.5 bc	7.2 a	7.3 a
11.4/0	1.2 b	1.4 d	0.8 f	2.8 bc	3.1 cde
11.4/4.6	4.2 ab	1.1 d	2.6 ef	1.8 c	4.4 abcde
11.4/13.9	3.0 ab	4.0 bc	5.2 cde	2.4 bc	5.1 abc
11.4/27.9	3.4 ab	2.6 cd	8.7 ab	4.6 abc	3.0 cde
11.4/46.5	7.8 a	6.7 a	10.7 a	7.2 a	5.1 abc
22.8/0	1.2 b	1.2 d	1.3 f	1.4 c	4.0 bcde
22.8/4.6	5.8 ab	1.3 d	3.2 def	1.8 c	5.1 abc
2.8/13.9	5.4 ab	2.1 cd	5.9 c	2.0 c	5.5 abc
22.8/27.9	1.8 b	2.9 cd	5.7 cd	3.4 abc	7.5 a
22.8/46.5	4.4 ab	5.4 ab	10.8 a	4.0 abc	6.4 ab

Values with the same letter are not different at 5 %, determined by the LSD test (Fisher test).

Table 5 – *In vitro* shoot regeneration in explants from *Echinocactus platyacanthus*, *Echinocereus pectinatus*, *Echinocereus schmollii*, *Ferocactus amatacanthus* and *Melocactus matanzanus* cultured on Murashige and Skoog (MS) medium containing combinations of indole-3-acetic acid (IAA)/kinetin (Kin) after 60 days in culture.

IAA/Kin (µM)	*Echinocactus platyacanthus*	*Echinocereus pectinatus*	*Echinocereus schmollii*	*Ferocactus amatacanthus*	*Melocactus matanzanus*
0/0	1.8 ef	1.2 e	0.8 e	1.0 ds	1.5 ef
0/4.6	4.9 ab	1.9 de	10.4 bcd	1.1 d	1.0 f
0/13.9	4.6 abc	8.8 ab	11.0 bc	4.6 bc	2.9 cdef
0/27.9	2.8 bcdef	4.4 bcde	12.0 bc	3.0 cd	3.1 cdef
0/46.5	1.7 ef	1.6 e	17.8 ab	6.2 ab	4.0 cdef
5.7/0	2.1 def	1.6 e	1.4 e	1.3 d	1.2 ef
5.7/4.6	2.4 cdef	1.2 e	4.0 cde	1.4 d	1.6 ef
5.7/13.9	3.5 abcde	6.4 bc	10.6 bcd	3.2 cd	5.8 abc
5.7/27.9	4.2 abcd	7.4 abc	12.4 bc	5.0 bc	7.3 ab
5.7/46.5	4.5 abc	6.6 bc	24.0 a	9.0 a	7.5 a
11.4/0	0.9 f	1.0 e	1.2 e	1.2 d	1.2 ef
11.4/4.6	2.6 bcdef	1.4 de	7.8 cde	1.1 d	2.2 def
11.4/13.9	2.8 bcdef	3.8 cde	4.8 cde	3.4 bcd	3.5 cdef
11.4/27.9	3.9 abcde	5.8 bcd	8.6 cde	5.6 bc	7.2 ab
11.4/46.5	1.8 ef	11.0 a	10.6 bcd	5.8 bc	5.8 abc
22.8/0	2.4 cdef	1.2 e	0.8 e	1.1 d	4.8 abcd
22.8/4.6	3.9 abcde	1.6 e	0.9 e	1.2 d	3.6 cdef
2.8/13.9	5.6 a	7.0 abc	1.6 e	1.3 d	4.3 bcde
22.8/27.9	4.0 abcde	3.8 cde	2.0 de	1.6 d	7.5 a
22.8/46.5	2.3 cdef	8.0 ab	7.4 cde	1.4 d	5.7 abc

Values with the same letter are not different at 5 %, determined by the LSD test (Fisher test).

Figure 2 – *In vitro* culture of four cactus species on Murashige and Skoog (MS) medium supplemented with 11.4/46.5 µM indole-3-acetic acid (IAA)/ kinetin (Kin). Top left, *A. myriostigma*; top right, *C. georgii*; bottom left, *C. senilis*; and bottom right, *C. straussi*.

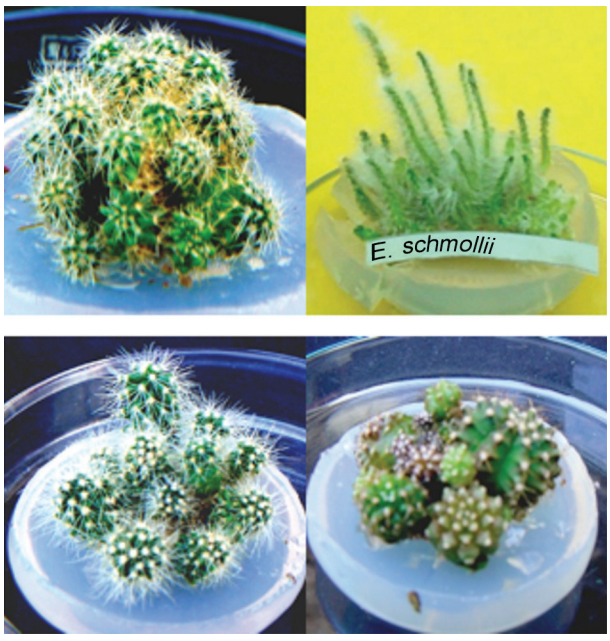

Figure 3 – *In vitro* culture of cactus species on Murashige and Skoog (MS) medium supplemented with 5.7/46.5 µM indole-3-acetic acid (IAA)/ kinetin (Kin). Top left, *E. grusonii*; top right, *E. schmollii*; bottom left, *F. amatacanthus*; and bottom right, *M. matanzanus*.

Similar results (10.8 shoots per explant) were also achieved for explants of *C. straussi* cultured on medium containing 22.8 µM IAA + 46.5 µM Kin (Table 4). In contrast, MS growth medium supplemented with 5.7 µM IAA and 46.5 µM Kin induced the highest shoot regeneration numbers in explants of *Echinocereus schmollii*, *Ferocactus amatacanthus*, *Melocactus matanzanus*, *Coryphanta georgii* and *Echinocactus grusonii* (Figure 3) with 24, 9, 7.5, 7.2 and 7.3 shoots per explant, respectively

(Tables 4 and 5). *Coryphantha georgii* explants showed similar responses (7.2 shoots per explant) on MS growth medium containing either 5.7/46.5 or 11.4/46.5 µM IAA/Kin (Table 4). Finally, *Echinocereus pectinatus* cultures attained maximum shoot formation per explant (11.0) on medium containing 11.4/46.5 µM IAA/Kin (Table 5). Rooting was recorded in 90 % of regenerated shoots and plant surviving efficiency during acclimation was 85 %.

Via somatic embryogenesis

Somatic embryogenesis is a pathway for the regeneration of plants that consists of the production of embryos from somatic cells. These embryos are able to germinate and produce a complete plant in a similar way to a zygotic embryo. However, since regenerated plants derive from somatic cells, they retain the genetic characteristics of the original plant. Somatic embryos can be produced directly on the explant or indirectly from calli. This is one of the most efficient ways for *in vitro* mass propagation of plants. However, many species are recalcitrant and very difficult or impossible to regenerate in this way. In the case of cacti, somatic embryogenesis has been reported among others for prickly-pear (*Opuntia ficus-indica* (L.) Mill.) (Ferreira-Gomes et al., 2006); for this species direct somatic embryogenesis was achieved by cultivating shoot apices devoid of leaf primordia on semisolid MS basal medium supplemented with 16.6 µM 4-amino 3,5,6-trichloropicolinic acid (picloram). Indirect somatic embryogenesis has been reported for *Schlumbergera truncata* (Haw.) Moran (Al-Ramamneh et al., 2006a). In this case, callus tissue was obtained on MS-based medium with 7 µM kinetin. Somatic embryos were induced when the callus was transferred onto MS-based medium with either 0.45 µM 2,4-D or without growth regulators (Al-Ramamneh et al., 2006a).

Miscellaneous applications of in vitro culture to ornamental cacti

Undoubtedly, mass propagation has been the most important objective of *in vitro* cactus culture. However, there are some other applications of this technology that could also contribute significantly to the improvement, conservation and rational use of these plants. Some of the applications that have already been reported for this group of plants are described below.

In vitro conservation of germplasm

An application of *in vitro* culture that can be very useful for the management and conservation of ornamental cacti is the preservation of living tissues for germplasm collections. However, the maintenance of viable cultures over long periods of time requires frequent subculturing, which generates labor and culture media costs. Furthermore, contamination risks due to manipulation occur during maintenance of subcultures. To avoid these disadvantages, slow growth systems have been described that aim to extend the time between subculturing (from a few weeks to several months) to facilitate and render less expensive the maintenance of viable cultures over long periods of time. To decrease the *in vitro* tissue growth rate, without affecting viability, controlled osmotic stress may be applied using chemical compounds in the growth medium, such as mannitol or sorbitol or most frequently high sucrose concentration, which reduce the osmotic potential without altering the biochemical balance of plant cells.

The growth rate may also be reduced by incubating the cultures at low temperatures or by using growth inhibitors such as abscisic acid, chlormequat chloride or n-dimethyl amino succinamic acid, in the growth medium. Also darkness, or green or yellow light is used for this purpose. Finally, a combination of two or more of the aforementioned treatments may be an efficient alternative for maintaining viable tissues under slow-growth conditions (Lynch, 1999; Sarasan et al., 2006). More recently, incubation in the presence of mineral oil has been proposed as another form to retard the *in vitro* growth of some plant species (Kulus and Zalewska, 2014). Slow-growth systems are relatively simple and a low-cost alternative for the conservation of plant germplasms. However, despite the advantages of this methodology, to our knowledge, there is only one report of its application to cactus species.

Pérez-Molphe-Balch et al. (2012) described an *in vitro* conservation technique employing slow-growth conditions in 16 threatened species and subspecies of the genus *Turbinicarpus*, which are native to the Chihuahuan Desert (México). The presence of osmotic agents, such as mannitol (30 g L^{-1}) and sorbitol (30 g L^{-1}), in the growth medium combined with low-temperature incubation (4 ± 0.5 °C) reduced the *in vitro* growth rate and did not affect shoot viability. The explants that were maintained under the described treatment conditions for 12 months were capable of regenerating shoots via areole activation when transferred to growth media containing 4.4 µM BA. Using this methodology, it has been possible to maintain an *in vitro* bank of viable tissues of these species with minimal maintenance and with the possibility of obtaining plants whenever wished. The Autonomous University of Aguascalientes (México) has used this technique to establish and maintain an *in vitro* germplasm bank comprising 122 Mexican cactus species (Figure 4A). All of these species have a high ornamental value, and the majority are considered threatened (Figure 4B-D). This technology provides specimen and tissue availability for different purposes without requiring their collection from the environment. One of the most important tools for the conservation of plant germplasm is cryopreservation (storage at -196 °C). However, in the case of cactus, this technique has only been applied to seeds, not to shoots or tissues cultured *in vitro* (Kulus and Zalewska, 2014).

Callus tissue: establishment and uses

Callus is an undifferentiated tissue that develops due to tissue injury and/or disorganized proliferation of cells. This tissue is easily obtained and maintained *in*

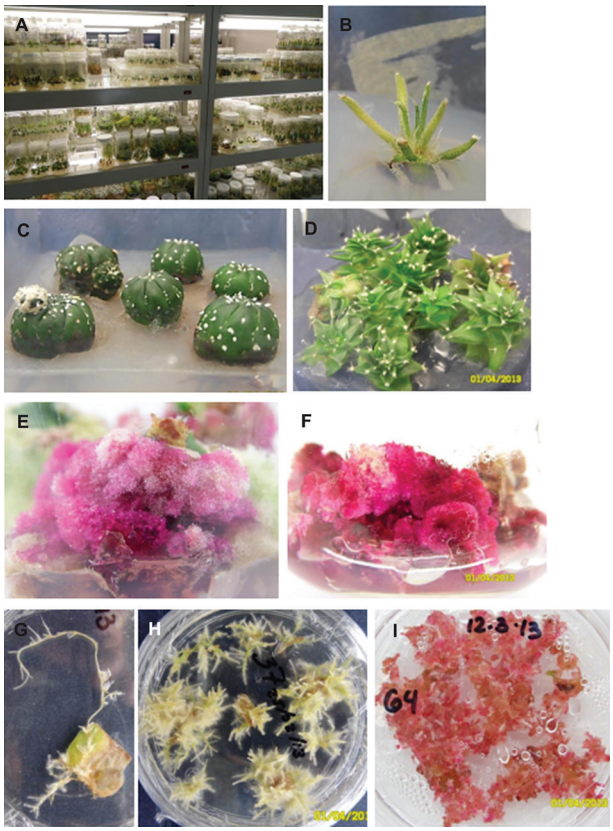

Figure 4 – Conservation of Mexican cacti in the germplasm bank of the Autonomous University of Aguascalientes (A); shoots of *Astrophytum caput-medusae* (B), *A. asterias* (C) and *Obregonia denegrii* (D) conserved in a slow-growth system; production of betalains from calli of *Myrtillocactus geometrizans* (E) and *M. schenckii* (F); generation of transformed roots from a leaf segment of the arboreal cactus *Pereskia sacharosa* (G); cultivation of transformed roots of *Mammillaria bocasana* (H); and transformed roots of *Turbinicarpus pseudopectinatus* producing betalains (I).

vitro and has been used as an intermediate step toward indirect plant regeneration via organogenesis or somatic embryogenesis. It is also used for basic studies in cellular physiology and biochemistry and for the *in vitro* production of secondary metabolites and can potentially be used as a source of new variation. To obtain callus tissue, segments or explants from stems, leaves, flowers or roots are cultivated in a nutrient medium containing high auxin to cytokinin ratios. For the maintenance and multiplication of callus tissues, fragment portions are transferred to fresh medium of the same composition.

The first attempt at *in vitro* mass propagation of cacti from callus cultures was reported by King (1957). A further report on the *in vitro* culture of cacti was the callus tissue generation and subsequent regeneration of entire *Mammillaria woodsii* plants (Kolář et al., 1976). These authors established the *in vitro* cultures by growing disinfected shoot sections on medium containing 11.4 µM IAA and 9.3 µM kinetin. Similarly, Mangolin et al. (1994) generated calli, which subsequently formed shoots in *Cereus peruvianus* (L.) Mill., using 18.1 µM 2,4-D and 18.6 µM Kin. In this study, they also analyzed isozyme electrophoretic patterns in the callus lines and in the regenerated shoots to investigate the possible variation induced by the culture process finding significant differences between the original material and the callus lines and plants regenerated from them two years later.

Karimi et al. (2010a) investigated the effect of genotype, explant size and position on the induction of callus tissue in *Cereus peruvianus* and found that apical and lateral explants responded better under all conditions tested, but apical explants showed the greatest callus formation, whereas the explant size did not affect callus induction significantly. Karimi et al. (2010b) also tested callus formation in stem explants of *Cereus peruvianus* bearing areoles or not and inoculated them on MS medium with combinations of NAA (5.37, 10.7, 16.1 and 21.5 µM), 2,4-D (4.52, 9.0, 13.6 and 18.1 µM), and Kin (4.6, 9.1, 13.7 and 18.3 µM), and observed higher callus formation in 18.1 µM 2,4-D in explants without areoles. In a more recent study, Karimi et al. (2012) investigated the seasonal effect on callus formation in explants of *C. peruvianus* (L.) Mill. var. *monstrosus* and var. *tortuosus* and observed greater responses in explants of var. *tortuosus* collected in summer and cultured on MS with 18.1 µM 2,4-D, whereas those from spring plants showed better callus formation in the presence of 21.5 µM NAA. In turn, Wakhlu and Bhau (2000) reported the generation of callus tissue in *Coryphantha elephantidens* (Lem.) Lem. using 9.1 µM 2,4-D and 2.3 µM Kin. Calli were organogenic and retained this capacity for several subcultures (18 months).

Somaclonal variation

The *in vitro* culture systems, particularly those involving the generation of callus tissue, may cause genetic or epigenetic alterations in plant cells. These alterations accumulate over culture cycles and may (or may not) appear in plants regenerated from these cells or tissues. This phenomenon is called somaclonal variation and is considered undesirable when trying to propagate plants massively while retaining the original features. However, somaclonal variation can be used to obtain new varieties of ornamental interest.

There are very few studies of variations in cacti caused by *in vitro* culture systems. Mangolin et al. (2002) used Random Amplified Polymorphic DNA (RAPD) markers to detect DNA polymorphisms in callus tissues of *Cereus peruvianus* cultured on media with different combinations of auxins and cytokinins. They observed that the greatest genetic variation occurred when the tissue was cultured in the presence of 18.1 µM 2,4-D and 37.2 µM Kin indicating that this medium could be used to increase variation to select cell lines that could produce higher levels of metabolites or have other features of interest.

Haploid plant generation

The use of haploid techniques to produce homozygous lines in a single step greatly accelerates the breeding process. Haploids and double haploids have been produced successfully from anthers or unpollinated ovules from several plant species; however, in the case of cactus there are few reports in the literature. One of the first attempts to induce haploid callus formation was reported by González-Melendi et al. (2005), who produced haploid callus tissue from anthers of *Opuntia ficus-indica* (L.) Mill. cultured on MS medium supplemented with 5 % sucrose, 0.1 µM NAA and 0.8 % agar; however, no plant regeneration was achieved under the conditions tested.

Benega García et al. (2009a) regenerated entire plants from anthers (androgenesis) of the vine cacti *Selenicereus megalanthus* (K. Schum ex Vaupel) Moran and *Hylocereus polyrhizus* (F.A.C. Weber) Britton & Rose. Anthers of *S. megalanthus* were cultured on MS basal medium supplemented with picloram (8.3, 16.6, 24.8, 33.1 and 14.4 µM) combined with BA (1.8 µM), while anthers of *H. polyrhizus* were inoculated on MS basal medium with TDZ (0.045, 0.23, 0.45, 2.3 and 4.5 µM) or 2,4-D (9.0, 18.1, 27.2, 36.2 and 45.2 µM). Embryos were regenerated from anthers to different extents on these media and complete plants were obtained subsequently; this was the first report on *in vitro* production of haploid cactus plants. Benega García et al. (2009b) also regenerated plants of *S. megalanthus* from unpollinated ovules (gynogenesis) collected from developing flower buds and culturing on MS basal medium containing 2,4-D/TDZ (2.27/2.27 µM) and sucrose at different levels (0-09, 0-18 and 0.26 M). No description of morphological differences between the haploid regenerated plants and the original explant donor was reported.

Embryo rescue

Embryo rescue is an *in vitro* application using the unviable/aborted seeds that typically result from hybridizations of incompatible plant species. In many cases, this technique allows for the generation of hybrid plants that are impossible to obtain using conventional methods. For this technique the fertilized ovule must be removed from the unviable or aborted seed and then cultured on a suitable medium to continue its development to generate an entire plant. One of the most important factors for this technique to be successful is to remove the embryo at the early stages of development.

Cisneros et al. (2013) established an *in vitro* system for rescuing embryos from interspecific-interploid crosses between *Hylocereus* sp. (A. Berger) Britton & Rose using a tetraploid *H. megalacanthus* (Vaup.) Bauer as the female parent and a diploid *H. monocanthus* (Lem.) Britton et Rose or *H. undatus* (Haw.) Britton et Rose as the male parent. Fertilized ovules were rescued 10 or 30 days after pollination and cultured on half-strength MS medium supplemented with 680 µM glutamine, 0.54 µM NAA and 0.45 µM thidiazuron, and containing 0, 0.09, 0.17 or 0.26 M sucrose. The greatest embryo development was achieved with 0.17 M sucrose. More than 70 % of the rescued hybrids were successfully acclimated and transplanted to soil. Diploidy, triploidy, tetraploidy and higher ploidy levels were detected in 77 putative hybrids, and from them 22 progenies were further randomly selected for Amplified Fragment Length Polymorphism (AFLP) analysis, and all were identified as hybrids. This technology results in novel, interspecific hybrids of vine cactus species.

In vitro production of plant metabolites

In addition to their ecological importance to arid climates, cacti produce many metabolites of interest. Alkaloids and betalain-type pigments (Figure 4E and F) are of potential use in the pharmaceutical and food industries. Many of these plants are very small in size and their rate of development is very slow. As an example of this slow development, it has been found that the saguaro, *Carnegiea gigantea* (Engelm.) Britt. and Rose, takes about 60 years to begin to form branches, and the reproductive stage is reached between 50 to 100 years, depending on the growth location (Drezner, 2014). These factors make their exploitation for metabolites very difficult. The alternative is to produce the metabolites using *in vitro* systems.

Oliveira and Silva-Machado (2003) reported the production of alkaloid amines in callus tissue cultures of *Cereus peruvianus*. Callus tissues were cultured in the presence of tyrosine, which is a precursor of the alkaloids tyramine and hordenine. The ratio of alkaloid production between tissues of a mature plant growing in the field and callus tissue cultured *in vitro* was 1:1.7. In a further study, Rocha et al. (2005) demonstrated that the greatest alkaloid production in long-term callus cultures of *C. peruvianus* was recorded when 200 mg L^{-1} tyrosine was added to the growth medium, and differential expression of some esterase isoenzyme genes was detected in the callus treated with tyrosine. These data demonstrated that callus tissue could be a viable source for metabolites of interest.

In turn, Santos-Díaz et al. (2005) established highly red-pigmented callus cultures from *Mammillaria candida* on MS media with the auxins 2,4-D (13.6 µM), NAA (16.1 µM), or chlorophenoxyacetic acid (16.1 µM) alone or in combination with the cytokinins Kin (4.6 µM), Kin riboside (2.9 µM), or benzyladenine (4.4 µM). Approximately 80 % of the calli were pigmented when grown on media containing auxins, whereas 50 % were pigmented when grown on media containing auxins and cytokinins. Tissue pigmentation levels had a 4-fold increase on media containing 50 or 100 g L^{-1} glucose or sucrose to cause abiotic stress, and with *Fusarium* sp. fungal extracts to simulate biotic stress, respectively. Spectrophotometric analysis revealed that the pigment produced was a modified betaxanthine. These studies showed that the main secondary metabolites produced by cactus could be obtained using *in vitro* cultured tissue, and opens the possibility of using bioreactors for the large scale production

of these compounds. However, to date no bioreactors with cells or tissues have been used for the production of cactus secondary metabolites.

Genetic transformation

One of the greatest achievements of plant biotechnology is the possibility of introducing new genes into the genome of cells, tissues or whole plants or silencing those already present. This technology has become one of the most interesting options for improving cactus crop plants. However, as with other applications of biotechnology, there has been little progress with cactus. In relation to regeneration of entire transgenic plants, Silos-Espino et al. (2006) developed a protocol for the genetic transformation of an elite prickly pear cactus (*Opuntia ficus-indica* L., cultivar Villa Nueva) by infecting it with *Agrobacterium tumefaciens* with the aim of using this technology to improve certain agronomic characteristics of the cultivated genotypes of this species. The transgenic character of the regenerated plants was demonstrated by both Polymerase Chain Reaction (PCR) and Southern blot analysis; transgene copy number in the transformed plants ranged from two to six, whereas the transformation frequency was 3 %. This method is recommended for routine transformations and the introduction of important or interesting genes into the prickly pear cactus. Al-Ramamneh et al. (2006b) reported the *Agrobacterium*-mediated genetic transformation of *Rhipsalidopsis gaertneri* ('Easter cactus'), an epiphyte native to forests in Brazil, using callus tissue induced from phylloclade explants and subcultured for a period of 9-12 months on MS medium with 22.7 μM TDZ + 1.3 μM NAA and then co-cultured with the LBA4404 strain carrying the selectable marker *nptII* gene and the reporter *uidA* gene. Selective conditions (600 mg L^{-1} kanamycin) were imposed for 9 months and adventitious transgenic shoots were recovered after kanamycin removal on MS growth medium supplemented with 0.62 μM daminozide. Transformation events were confirmed by GUS staining, ELISA analysis and Southern blot hybridization for the *nptII* gene. A transformation efficiency of 23 % was achieved. More recently, an *in planta* transformation protocol of *Notocactus scopa* by *Agrobacterium tumefaciens* (LBA4404) vacuum infiltration combined with pin-pricking was described by Seol et al. (2008) with efficiencies of 67-100 %. Expression of *uidA* and *nptII* genes was detected in the regenerated plants.

Another variation of genetic modification of plant tissues is the production of transformed roots, also called hairy roots (Figure 4G). In this case, the bacterium *Agrobacterium rhizogenes* is used. The importance of transformed roots lies in their potential utilization as a source of metabolites, alkaloids and betalains for example, due to their biosynthetic potential and high rate of development *in vitro* (Figure 4H and I). In the case of cactus, the only published study is of González-Díaz et al. (2006) who evaluated the susceptibility of 65 species of 22 genera of Mexican cacti to *Agrobacterium rhizogenes*. Stem discs from *in vitro* cultured plants were infected with a suspension of the A4 agropine-type strain of *Agrobacterium rhizogenes* bearing the wild RiA4 plasmid and the binary vector pESC4 with the *nptII* and *gus* genes. Hairy roots were induced directly from wounds or were generated from calli produced on the wounded surface in 34 (52 %) of the evaluated species. Hairy-root formation frequencies varied from 3 to 87 % among the species tested. The number of transformed roots also varied in the range from 1 to 26 per explant. Formation of transformed roots in the 31 remaining species was not observed under the conditions tested. Expression of the foreign gene in the hairy roots was detected by histochemical assays of β-glucuronidase (GUS) activity; furthermore, the presence of the *rolB* and *nptII* genes in the DNA of the transformed roots was demonstrated by PCR analyses.

Other studies

In vitro cultures of cacti have also been used as model systems for basic studies; for example, research into the CAM photosynthetic metabolism of these plants. Malda et al. (1999a) noted an increase in the net photosynthetic rate (carbon assimilation) in *in vitro* plants of *Coryphantha minima* Baird compared with those maintained in *ex vitro* conditions. They explained this result as being due to a difference in net CO_2 uptake by the *in vitro* cultured plants, which occurred continuously in both the light and the dark, compared with the CAM plants grown in *ex vitro* conditions. This factor, together with high relative humidity, nutrient-rich culture medium and the presence of hormones, induced the considerably faster development of cacti cultured *in vitro* (seven times faster than similar plants grown *ex vitro*). Balen et al. (2012) reported that *in vitro* conditions affected photosynthetic performance and crassulacean acid metabolism in *Mammillaria gracilis* Pfeiff. tissues since reduced photosynthesis in *in vitro* grown habituated callus, hyperhydric shoots and tumor tissue were detected, but not in *in vitro* grown normal shoots.

Final Remarks

Cactaceae members are greatly appreciated as ornamental plants worldwide. In general, cacti are very well adapted to growth in arid or semiarid regions where water availability is very limited. However, typically, they grow very slowly. Thus, the time required for their multiplication and commercial availability to customers is so long that in many cases, the natural populations are over-collected and over-exploited causing dramatic reductions leading to many species being threatened, endangered or brought to the brink of extinction. Tissue culture techniques have been applied as an alternative to reduce the propagation time. To date, more than 100 cactus species have been successfully propagated *in vitro*. The interest of researchers has been focused on those species that have agricultural importance (e.g.: the genera *Opuntia* and *Hylocereus*), species with high orna-

mental value (*Schlumbergera*) and endangered species. In the last group, what stands out are the successful reports of species of the genera *Coryphantha*, *Mammillaria*, *Pelecyphora* and *Turbinicarpus*. They fully prove that *in vitro* propagation can be the more efficient alternative for the rescue of such species. A case that exemplifies this is *Mammillaria san angelensis*, species endemic to a small region within the Federal District, Mexico, which was rescued from imminent extinction through *in vitro* propagation (Martínez-Vázquez and Rubluo, 1989) and can now even be acquired from commercial nurseries. Although there are reports of *in vitro* propagation of cacti through organogenesis and somatic embryogenesis, the most used pathway has been the multiplication from areoles. For this, the only requirement is the use of explants containing areoles and culture on a medium supplemented with cytokinins. The cytokinin which has yielded the best results has been benzyladenine (BA).

Future research

Most cacti can be propagated relatively easily by seeds. Unfortunately, in most species seed availability is insufficient to produce the number of plants required for marketing or for use in reintroduction programs. In these cases asexual propagation may be very useful. Tissue culture should be an important tool for cactus vegetative propagation. Thus far, a limited number of cactus species have been micropropagated for commercial purposes, and in some other cases, the primary aim has been the multiplication of endangered or threatened species to restore their populations in the wild. Therefore, future research should focus primarily on the establishment of micropropagation protocols for a large number of cactus species that must be propagated for commercial or ecological reasons. Tissue culture techniques should be of great value for conserving and preserving cactus genetic resources primarily for endangered or threatened species; this work should be facilitated by the fact that, in general, *in vitro* cultures from cacti typically show slower growth than those from other plant species and, therefore, do not need frequent subculturing. Some other potential biotechnological applications of cactus tissue culture, such as haploid and double-haploid plant generation and genetic modification by genetic engineering, appear to have not been widely explored thus far. Isolation, characterization and production of secondary metabolites by organs, tissues or cell cultures from cactus species are currently very limited and should also be extended to obtain knowledge of their potential uses.

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